SPECIFIC IMMUNE RESPONSE AND TOLERANCE IN MICE TRANSGENIC FOR HETEROLOGOUS PROTEINS SECRETED IN MILK.

Lesley Ann Smyth

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF SCIENCE, UNIVERSITY OF EDINBURGH, 1995

ABSTRACT

The expression of the ovine milk protein β-lactoglobulin (BLG) in transgenic mice is both mammary specific and hormonally regulated only being produced in mammary epithelial cells during lactation and gestation. Since BLG is a protein not encoded in the rodent genome this transgenic system presents a unique opportunity to investigate systemic tolerance to a 'foreign' transgenic protein and neonatal tolerance to a milk protein encountered in the gut.

Systemic tolerance to BLG was studied in a) male and female mice heterozygous for the BLG transgene derived from crossing homozygous BLG transgenic males and wild-type females, b) BLG-transgenic and non-transgenic offspring derived from mating male and female heterozygous for BLG transgene with wild-type partners and c) BLG-transgenic and non-transgenic mice derived from back crossing onto a CBA/Ca MHC background. Using either a BLG-specific ELISA (for antibody responses) or footpad thickening assay (for T cell responses) the immune response to the BLG antigen was assessed. Hyporesponsiveness to both ovine and bovine BLG was observed at the antibody level but not at the T cell level for mice transgenic for BLG as compared to wild-type and non-transgenic littermates. Antibody tolerance could not be attributed to expression of the gene during pregnancy and lactation since all mice tested were virgin mice. These experiments also confirm that suckling on BLG-containing milk was not responsible for the antibody hyporesponsiveness seen in BLG-transgenic mice.

Male and female mice heterozygous for the BLG transgene were mated to wild-type partners such that the offspring fell into eight classes: male and female, suckled or non-suckled on 'transgenic' milk and heterozygous or wild-type for the transgene. Antibody and T cell data indicated that suckling "transgenic' milk did not induce oral tolerance to ovine BLG in either transgenic or non-transgenic offspring. In contrast, voluntary ingestion of bovine BLG by wild-type mice for 24 hour or 21 days resulted in both antibody and T cell tolerance.

Hyporesponsiveness could not be induced by transferring transgenic marrow into lethal irradiated normal recipients, but similarly irradiated transgenic recipients were still hyporesponsive after reconstitution with normal bone marrow.

These data demonstrate that in the absence of detectable expresssion of transgenic BLG protein mice are tolerant to this transgenic product at the antibody level but probably not at the T cell level. Bone marrow transplantation experiments have demonstrated that transgenic B cells can mount normal antibody responses to BLG in a wild type recipient environment, but wild type B cells tend to be tolerant to BLG immunisation in a transgenic environment. Although BLG can be demonstrated to be an oral tolerogen, wild type mice suckling on BLG-containing milk from birth are not tolerant to this antigen in adulthood.

In summary, some yet undetectable low level expression of a transgene, probably in a lymphoid differentiation environment, can render animals tolerant to the transgene antigen. Exposure to such antigens perinatally, through suckling, does not necessarily render offspring unresponsive to that antigen over the long term.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the following people;

1). My supervisors Dr. John Ansell and Professor Spedding Micklem for offering me this PhD position and for their support and help throughout the last few years.

2). Dr John Ansell for reading my thesis and for the whitty comments about my spelling and grammer (honestly, I did pass Higher English) and for putting up with me for so long.

3). Kay Samuels for all her help with the mouse work (sorry the F1 BLG-transgenics were a bit wild) as well as the personal advice throughout my PhD.

4). John Tweedie and all the staff in the animal house for looking after all my mice and for putting up with the halothane machine and its faults.

5). Drs John Clark and Paul Simons for the BLG-transgenic mice

6). Dr M McClenaghan and staff of the Roslin Institute farms for kindly helping with the milking operation.

7). Clive Lunny for various things, too numerous to mention but including reading of my thesis.

8). Tom Gardner for the photos.

9). Everyone on the second floor for putting up with my moods, tempers and bitching once the PhD write-up began.

10). And lastly I would like to thank my parents (and Shandy "The Dog") for their support and encouragement throughout my PhD studies especially at the beginning. Without their encouragement I would not have come this far.

I would also like to thank the SERC for funding my studentship as well as the Moray Fund for their financial support in my first year of study.

ABBREVIATIONS

ACHR - nicotinic acetylcholine receptor
ALB - albumin
APC - antigen presenting cell
BLG - β-lactoglobulin
BM - bone marrow
β_2m - beta-2 microglobulin
BSA - bovine serum albumin
C5 - the fifth component of complement
CETS - clusters of epithelial staining
CD - cluster of determinants
CM - corticomedullary junction
Con A - concanavalin A
CRP - c' reactive protein
CTL - cytotoxic T cell
CYT - cytoplasmic
3D - three dimensional
Dbl-tg - double transgenic mice
DC(s) - dendritic cell(s)
D_H - D segment of the immunoglobulin heavy chain
dH_2O - distilled water
DNA - deoxyribonucleic acid
ds DNA - double stranded
DN - double negative thymocytes
DP - double positive thymocytes
DTH - delayed type hypersensitivity
EAE - experimental autoimmune encephalomyelitis
EAU - experimental autoimmune uveitis
ELISA - enzyme linked immunosorbant assay
EM - electron microscope
FACS - fluorescence activated cell sorter
FCA - complete Freunds adjuvant
FDC(s) - follicular dendritic cell(s)
FICA - Freunds incomplete adjuvant
Fig (s) figure(s)
FTOC - fetal thymic organ culture
GFAP - glial fibrillary acid protein
GM-CSF - granulocyte monocyte colony stimulating factor
HA - influenza virus hemagglutinin
HEL - hens egg lysozyme
HGG - human gamma globulin
HIP - human insulin promoter

HRP - horse radish peroxidase
HPRT - hypoxanthine guanine phosphoribosyl transferase
HRBC - horse red blood cells
HSA - heat shock antigens
HSA - human serum albumin
ICAM - intercellular adhesion molecule
IF - influenza virus
IFN γ - interferon gamma
Ig -immunoglobulin
Ig-T - anti-HEL Ig transgenic mice
IL - interleukin
IL-2Rα - interleukin 2 receptor α chain
IP - intraperitoneally
IV - intravenously
J_H - J segment of the immunoglobulin heavy chain
LPS - lipopolysaccharide
kDa - kilodalton
KIV - keratinocyte
KLH - keyhole limpet haemocyanin
LCMV - lymphocytic choriomeningitis virus
LFA - leucocyte functional antigens
mIg - membrane immunoglobulin
MBP - myelin basic protein
MGF - mast cell growth factor
MHC - major histocompatibility complex
MLR - mixed lymphocyte reaction
Mls - minor lymphocyte stimulation antigen
MMTV - mouse mammary tumor virus
mRNA - messager RNA
NOD - non-obese diabetes
NP - nucleoprotein
OD - optical density
ORF - open reading frame
OVA - ovalbumin
PBS- phosphate buffered saline
PC - phosphatidyl choline
PCR - polymerase chain reaction
PFC - plaque forming cells
PHA - phytohaemagglutinin
PKC - protein kinase C
PLN - peripheral lymph node
PGE_2 - prostaglandin E2
RAG - recombinase activation genes
RBC(s) - red blood cell(s)

RBP - retinol binding protein
RF - rheumatoid factor
rIL-2 - recombinant interleukin 2
RIP - rat insulin promoter
RNA - ribonucleic acid
RT-PCR- reverse transcriptase PCR
SC - subcutaneously
SCID - severe combined immunodeficiency syndrome
SD - standard deviations
SEA - staphylococcal enterotoxin A
SEB - staphylococcal enterotoxin B
SDS- sodium dodecyl sulphate
sIg - secretory immunoglobulin
SLE - systemic lupus erythematosus
SRBC - sheep red blood cell
ss DNA - single stranded DNA
STI - soyabean trypsin inhibitor
SP - single positive
TAP- transporter associated with antigen processing
TCR - T cell receptor
Tdt - terminal deoxynucleotidyl transferase
tg - transgenic
TGFβ – transforming growth factor β
T_H - T helper cell
TM - transmembrane
TNC - thymic nurse cell
TNFα - tumor necrosis factor α
TNP - trinitrophenyl
VCAM- vascular cellular adhesion molecule
VLA - very late antigen
VSV-GP - vesicular stomatitis virus glycoprotein
WAP- whey acidic proteins
WBC - white blood cell
W+ - mouse diet containing whey protein
W- - mouse diet lacking whey protein

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

1.1) B cell differentiation

B cell differentiation has been reviewed extensively elsewhere see [1-18] and will be summarised here. B cells provide the humoral arm of the immune system through the expression of surface immunoglobulin (Ig) which acts as a specific antigen receptor. This receptor consists of two light and two heavy chains which are non-covalently linked to the products of the mb-1 and B29 genes, Igα (CD79a) and Igβ (CD74b) respectively. Igα and Igβ are glycoslyated and phosphorylated transmembrane proteins with extracellular Ig like domains and cytoplasmic tails and they are involved in transporting Ig to the cell surface. The Igα and Igβ heterodimer is found associated in the mouse with IgM, IgD, Ig2b, IgG and IgE constant regions.

Signalling through this Ig receptor occurs following cross linking with a specific antigen. In mature B cells this results in proliferation and immunoglobulin secretion. In immature B cells this event may induce cell death.

Genes encoding the Ig light and heavy chains are present in the germline of lymphoid progenitors. There are two possible light chains, κ and λ, κ is the most common. In κ negative transgenic mice λ light chains are utilised instead; thus rearrangement of κ is not a prerequiste for the initition of λ rearrangements. Light chain genes are encoded by variable (V) and joining (J) genes, whilst heavy chains (H) are encoded by V, diversity (D), J and constant (C) genes. The C genes encoding the heavy chain/the isotype of the immunoglobulin, are μ (IgM) δ (IgD) γ (IgG) or α (IgA). Gene recombinase systems (terminal deoxynucleotidyl transferase (Tdt) and recombinase activation genes (RAG) 1 and 2) facilitate gene rearrangement leading to a functional gene product. Heavy chain rearrangement occurs first. One of many D segments is combined with a J segment

Fig. 1.1. B Cell differentiation

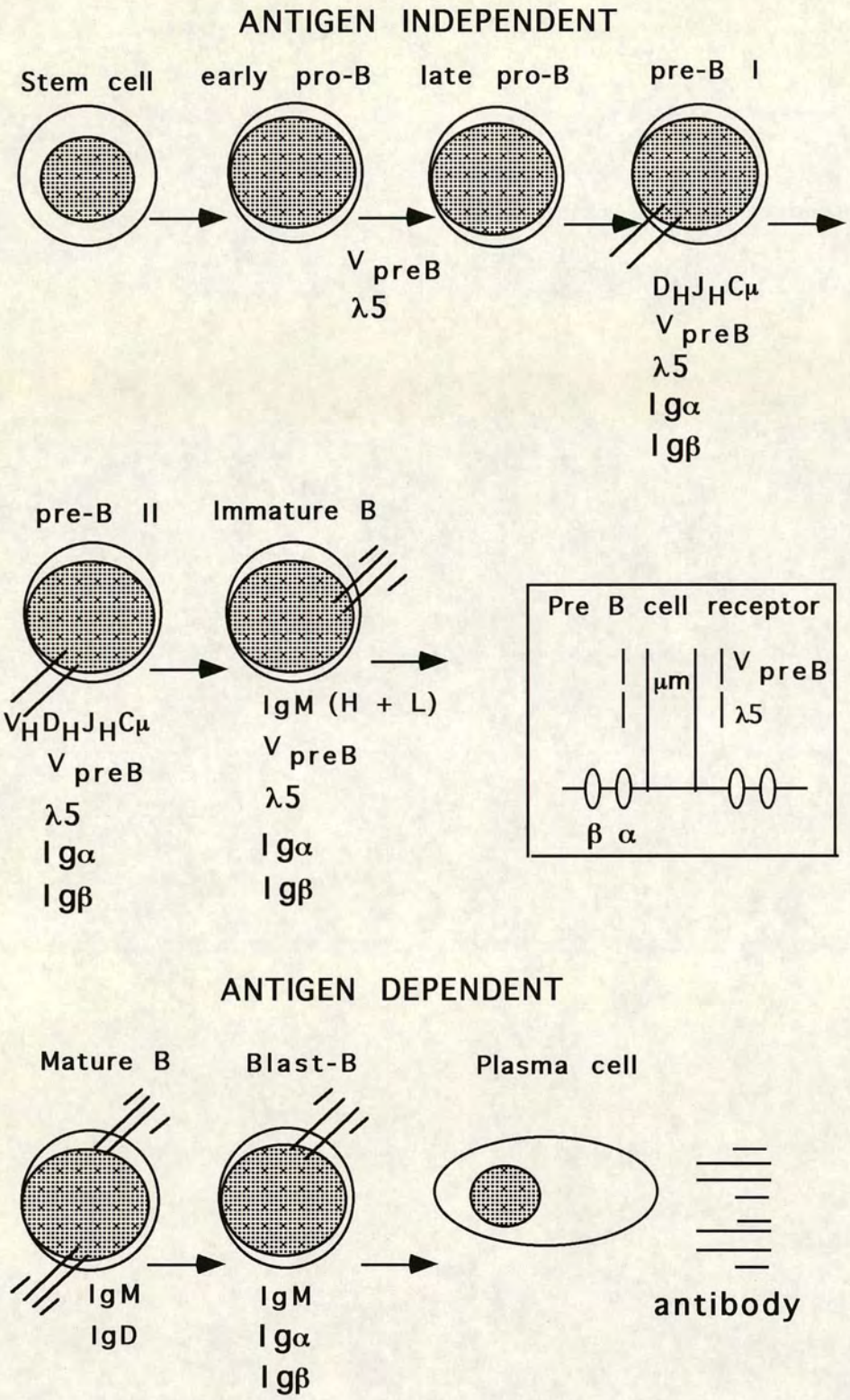
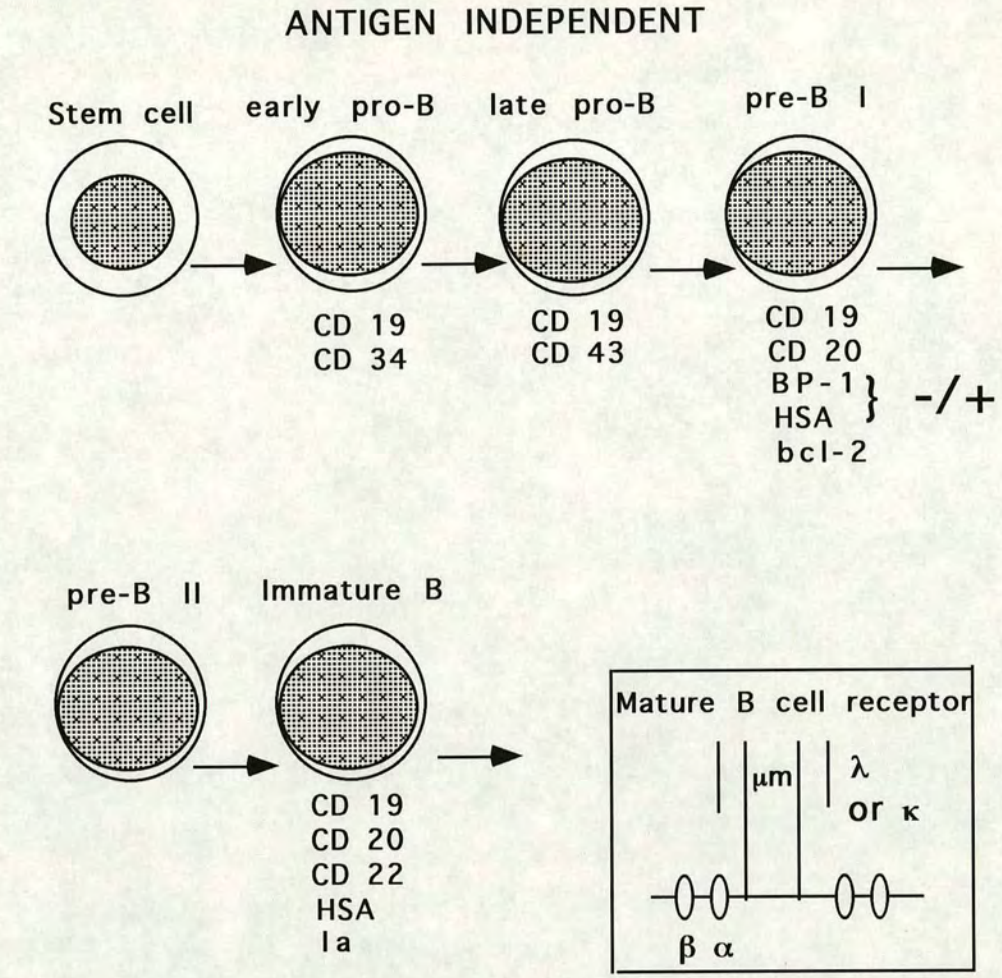


Fig. 1.2. B Cell differentiation



and this DJ combines with a V region. This VDJ then combines with the IgM heavy chain constant region. Following each of these combinations other V, D, and J regions are removed. Light chain rearrangement involves a V and J combination but unlike the heavy chain this does not alway result in the loss of the unused V and J regions. A large amount of diversity is possible due to the number of V, D and J region combinations. Diversity is also increased by the Tdt recombination system which adds N-sequences at the junction of these segment combinations.

B cells are derived from B cell committed lymphoid progenitor (stem cells) and the site for B cell differentiation is the bone marrow. This stage of development is independent of antigen, in other words antigen does not affect maturation of B cells within this primary lymphoid tissue.

The first stage in B cell development is characterised by the upregulation of "early" B cell lineage surface antigens, B220 in mice, and the machinery for immunoglobulin rearrangement. Each stage of the antigen independent phase of B cell differentiation has been named; the pro-B cell stages (early and late), pre-B cell stage (large pre-B cell I and and small pre-B cell II) and the immature B cell stage (see Figs 1.1 and 1.2).

Early pro-B cells express cell surface markers B220+, CD34+ and CD19+ whilst late pro-B cells express B220+, CD19+ and CD43+. These cells have immunoglobulin genes in germline configuration and they are further identified as belonging to the early B cell lineage due to expression of other B cell specific genes including mb-1, B29, $\lambda 5$ and $V_{pre\text{-}B}$ (surrogate light chains) in germline configuration. It has been shown in RAG -deficient mice that pro- B cells can express surrogate light chains without the IgM heavy chain [6]. At this stage of development pro-B cells express the immunoglobulin recombination genes Tdt and RAG 1 and 2.

The pre-B cell stage is split into 2 stages: pre-B cell I and pre-B cell II. At the pre-B cell I stage D_H and J_H recombination has occurred as well as recombination with the $C\mu$ heavy chain.

This $D_HJ_HC\mu$ complex is found on the cell surface in association with the non-covalently linked $\lambda 5$ and Vpre-B gene encoded surrogate light chains. At the pre-B cell I stage $Ig\alpha$ is expressed associated with $Ig\beta$. The $D_HJ_HC\mu/\lambda 5/V_{pre\text{-}B}/Ig\alpha$ and $Ig\beta$ complex is known as the pre-B cell receptor [2] This pre-B cell receptor is also expressed on pre-B cell II cells. However the $D_HJ_HC\mu$ is replaced by a functional $V_HD_HJ_HC\mu$. These cells do not undergo conventional light chain VJ rearrangements.

The importance of the $C\mu$ chain has been shown in transgenic homozygous mice carrying an inactivated exon of the μ (IgM) heavy chain resulting in mice lacking membrane μ heavy chain (Kitamura et al (1991) cited in [17]). These mice are devoid of mature B cells and they are arrested at the pre-B cell stage. Further evidence that a successful rearrangement of the IgM heavy chain is important for further B cell differentiation was shown using RAG deficient mice [6]. In these mice inactivation of the RAG 1 and 2 genes resulted in the complete absence of both mature B and T cells due to no initiation of the IgM heavy chain VDJ rearrangements in the case of B cells. B cell development was arrested at the pro-B cell stage. The appearance of pre-B cells in the bone marrow only occurred if a functionally assembled human μ or murine IgM+D+ transgenes were introduced into the RAG-1 or a RAG-2 deficient background respectively. No differentiation occurred if functionally assembled κ or λ light chain genes were introduced [6].

In these mice it was also shown that IgM heavy chain expression appeared to induce heavy chain allelic exclusion and conventional light chain gene rearrangement.

Evidence for the importance of the pre-B cell receptor was shown in transgenic mice in which the $\lambda 5$ and J_H genes were disrupted [17]. In these mice B cell development was also diminished. It was concluded that only pre-B cells expressing a $C\mu$ chain would express a pre-B cell receptor and this permits further differentiation.

As with pro-B cells, pre-B cells can express V_{preB} and $\lambda 5$ surrogate light chains in the absence of the $C\mu$ chain. However

expression of these chains requires association with a complex of proteins.

Pre-B cells also express various cell surface markers as shown in Fig. 1.2.

Immature B cells rearrange light chain genes and the IgM heavy chain is found associated with either κ or λ light chains. Surrogate light chains are still present on immature B cells. Igα and Igβ are also found associated with the IgM receptor. At this stage RAG and Tdt gene expression is turned off.

Immature B cells become virgin B cells which express IgM and IgD and these mature B cells no longer express surrogate light chains. B cell development at this stage is antigen dependent. During an immune response mature B cells are recruited into germinal centers (GC). In the presence of antigen on the surface of GC follicular dendritic cells (FDC) and with T cell help B cells proliferate and further differentiate into plasma cell/antibody forming cells. During this T cell dependent response virgin B cells undergo a process known as somatic mutation. Somatic mutation affects the V regions of the Ig receptor resulting in receptors with different specificities and affinity. Those B cells with high affinity receptors are selected for and rescued from apoptotic cell death. This process appears to involve the expression of bcl-2 which has been shown to prolong B cell life span. Transgenic mice expressing the bcl-2 gene under the control of the Ig heavy chain enhancer have more B cells than normal mice but these cells are non-dividing. Thus bcl-2 prevents cell death but does not promote B cell proliferation. Antibody production in these mice is greatly prolonged and mice eventually develop an systemic lupus erythematosus (SLE)-like autoimmune disease (Strasser et al (1991) cited in [17]).

These high affinity B cells differentiate into either plasma cells, the terminal differentiation point or into memory B cells which respond quicker and produce high affinity antibodies of different isotype following a secondary challenge with antigen. It is thought that the prolonged presence of antigen on the surface of FDC is important for the

maintainence of memory. It is also suggested that memory cells may require bcl-2 expression to be safe from apoptosis.
Various external factors have been implicated in B cell differentiation at both the antigen independent and dependent stages. Maturation is dependent on the interaction between the developing cell and bone marrow and peripheral lymph node stroma as well as with soluble factors. Developing B cells themselves and stromal cells express surface molecules which have been shown to influence maturation, for example, pro and pre-B cells express VLA-4 the ligand for which is VCAM-1 present on bone marrow stromal cells. Monoclonal antibodies to both inhibits binding of murine B cells to stromal cells and addition of anti-VLA-4 antibodies to long term bone marrow cultures prevented B cell lymphopoiesis (Miyakeit et al and Nuccie, Ryan and Abbound cited in [16]).
In mature B cells CD40 binds to its ligand on T cells and this is important for the generation of T cell dependent humoral immune responses. Treatment with anti-CD40 monoclonal antibodies prevent primary and secondary T dependent responses (Foy et al cited in [16]).
Mature B cells also express CD80 (B7/BB1) which is the ligand for CD28 present on T cells. In CD28 deficient mice IgM (T independent) responses are normal but IgG (T dependent) responses are deficient. Thus signalling via the CD28/CD80 is important for class switching. It has also been shown that the B7 molecule is not important for early B cell development since B7 deficient mice have apparently normal B cell populations (Freedman et al (1993) cited in [34]).
Mature B cells also express CD23 and CD21. CD23 is the ligand for CD21 which is present on the FDC.
Cytokines have also been shown to be important in B cell development. Il-7 plus stromal cells promote the preferential expansion of pre-B cells from suspensions of murine bone marrow and fetal liver cells [18]. Stromal cell derived cytokines have also been implicated in B cell maturation. Two cytokines IL-11 and mast cell growth factor (MGF) have been

defined although, they cannot overcome the requirement for stromal cells.

1.2) B cell tolerance

Possibily the easiest explanation for B cell tolerance is that it is a consequence of T cell tolerance. Due to the lack of T cell help and appropriate T cell signals, the B cell compartment is unable to produce auto-antibodies [19]. However the story is not as simple as this since self reactive T cells have been shown to escape from the thymus [20] and these cells have the potential to stimulate autoreactive B cells. Why is there a need for B cell tolerance? Unlike T cells, mature B cells with low affinity receptors on encountering both antigen and T cell help undergo a process called somatic mutation [21]. This process results in mature B cells secreting high affinity antibody and it may also result in self-reactive B cells [22]. Low affinity B cells expressing IgM receptors for self antigens have been found in normal mice at high frequency and these are non-tolerised (Primi et al (1987), Nakamura et al (1981), Holmberg et al (1986), Ternynck et al (1986) cited in [23]).
Through the use of transgenic (tg) mice B cell tolerance has been shown to occur for both immature and mature B cells. Tolerance appears to be operating through various routes either by deletion, silencing or functional arrest of self-reacting B cell clones according to the particular mouse model studied.

i) Deletion

a) Anti-H-2 K^k transgenic mice

Nemazee and Burki (1989) [24, 25] have created transgenic mice expressing an IgM antibody (3-83) which has an IgG idiotype specific for MHC Class $1K^k$ and the heavy chain of an IgM molecule. This antibody recognises MHC Class 1 K^k with high affinity and also recognises with low affinity (100-fold lower), MHC Class 1 K^b.
In transgenic mice with an $H\text{-}2^d$ background, the 3-83 IgM antibody makes up 61.6% of the total IgM. In transgenic mice

on an H-2^d x H-2^k (H-$2^{d/k}$) background the 3-38 IgM levels in the sera are reduced to approximately 15% of total IgM. In the spleens of the H-$2^{d/k}$ transgenic mice neither resting B cells nor plasma cells were present whereas on a non-selecting background 3-38 antibody producing cells were in the majority. Tolerance was also observed in the presence of the lower affinity K^b antigens.

This tolerance was not due to the expression of H-$2K^k$ by the anti-H-$2K^k$ B cells themselves as shown by transferring H-2^d bone marrow to irradiated H-2^d X H-2^k, or H-2^d recipients. 3-83 IgM antibodies were found in mice expressing the H-2^d but not the H-2^d X H-2^k mice.

These experiments suggested that the site of tolerance in these mice was the bone marrow [24]. Further experiments supported these conclusions. Bone marrow depleted of T cells from an anti-H-$2K^k$ transgenic mouse was transplanted into both H-2^d X H-2^k or H-2^d mice. Only in the presence of H-2^k were the 3-83 IgM antibodies deleted from the spleen. However some anti-H-$2K^k$ idiotypic bearing pre-B cells, expressing B220 and membrane (m)IgM were found in the bone marrow of H-2^d X H-2^k transgenic mice, but in reduced numbers. The presence of self-reactive B cells which appear to escape tolerance is due to receptor editing [26-35].

These data led to the hypothesis that tolerance is affecting the pre-B cell stage of B cell ontogeny and that the mechanism is one of a deletion rather than a down regulation of Ig receptors.

These transgenic mice have been further used to investigate autoimmunity in MRL-lpr/lpr mice [36], which contain B cells with specificities for DNA. Crossing anti-H-$2K^k$ 3-83IgM transgenic mice with either a H-2^k or H-2^d MHC background with MRL-lpr/lpr mice (H-$2K^k$) resulted in transgenic mice expressing both the 3-83 transgene on a background of either H-2^k or H-2^d X H-2^k. These mice had significant numbers of 3-83 IgM positive B cell and low levels of 3-83 in the sera. These B cells were not anergic and secreted the transgene encoded IgM antibody. Control, non MRL-lpr/lpr mice, on these

backgrounds gave results similar to the data above, suggesting that the autoimmune disease of the MRL-lpr/lpr mouse is due to a failure in deletion of self-reactive B cells.

b) Hen egg lysozyme (HEL) - transgenic mice

Deletion was also shown to be responsible for tolerance in double transgenic (Dbl-tg) mice expressing both the HEL transgene (under the control of the H-2K^{b} promoter) and either IgM+D+ or IgM+ transgenes with anti-HEL specificity [37]. In the HEL-only transgenic mice HEL is expressed as a membrane bound protein on the surface of B cells found in the spleen, bone marrow, lymph node and thymus.

Dbl-tg mice do not make anti-HEL specific antibody. This appears to be due to the deletion of lysozyme binding B cells, since FACS analysis of both spleen and lymph node indicated the absence of mature anti-HEL Ig binding cells in these mice. This observation was not affected by the absence or presence of IgD.

Deletion of mature B cells in these mice was also shown via bone marrow chimaeras: irradiated HEL expressing transgenic mice were recontituted with anti-HEL Ig (IgM+D+) bone marrow. The recipient mice were devoid of B220^{hi} B cells bearing the HEL specificity. However there was no reduction in the number of B220^{lo} mIg+ve cells in the bone marrow. This agreed with the anti-H-2K^{k} transgenic mice and further suggested that deletion of these cells occurs at the pre-B cell to immature B cell stage of development. The reduced levels of mIgM on these cells suggested that they had encountered antigen although this exposure had not induced cell death since no sign of apoptosis was found. This led the authors to suggest that deletion occured in two steps. The first step, functional arrest is caused by the interaction of immature cells and membrane bound antigen and the second, and later step, cell death [38].

Arrested development results in immature B cells which express low levels of membrane IgM and which do not acquire adhesion, activation and migration receptors. This step is

reversible since exposure to membrane bound HEL antigen did not result in a cell incapable of response. Sorted B220^{lo} IgD-ve bone marrow B cells from the Dbl-tg mice, when cultured with thymocytes not expressing the HEL protein, expressed a twenty fold increase in mIgM+ levels and an increase in B220 and IgD expression: ie; in other words a more mature B cell phenotype. This did not occur when they were cultured with thymocytes from an HEL tg mouse.

Cell death, which was shown to occur after 1-3 days when Dbl-tg cells were constantly exposed to HEL, could be prevented by the introduction of the bcl-2 transgene. This protein has been shown to greatly increase the life span of both immature and mature B cells in transgenic mice expressing bcl-2 under the control of an Ig heavy chain promoter. Bcl-2 transgenic mice were mated with anti-HEL Ig transgenic mice. Bone marrow from these (bcl-2 x anti-HEL) transgenic mice was transferred into irradiated HEL transgenic and non-transgenic mice. Control mice were given anti-HEL Ig bone marrow. Non-transgenic mice that received anti-HEL bone marrow had mature B cells expressing high levels of B220 as well as homing and adhesion molecules whilst HEL transgenic mice receiving this bone marrow did not express mature B cells in the spleen, lymph node or peripheral blood. However when HEL transgenic mice were given (bcl-2 x anti-HEL Ig) bone marrow a large number of B220^{lo} CD23-ve, CR1 and 2-ve, IgD-ve, CD22-ve and L-selectin -ve immature B cells were present in peripheral blood and spleen. These B cells also expressed large amounts of anti-HEL IgM and were found to be self-reactive in that they bound HEL. An immature to mature B cell phenotype transition was achieved when the HEL antigen was removed. Mature HEL binding cells also occurred in non-transgenic mice reconstituted with this (bcl-2 x anti-HEL Ig) bone marrow combination.

From these data it was concluded that the deletion process occurred in two independent steps within the bone marrow. Expression of the bcl-2 gene stops cell death but does not interfere with the arrested development such that immature B

cells are present in these mice. The persistent expression of HEL antigen however does not allow phenotypic maturation of these cells, and in the absence of the bcl-2 gene would result in cell death. Removal of the antigen allows mature cells to develop. These data were supported by the use of the immature B cell lines CH31 and WEHI123. It was also found that IL-5, as well as bcl-2, could inhibit anti-IgM induced apoptotic deletion while not affecting the arrested development stage [39].

c) Autoimmune Haemolytic Anaemia (AHA) transgenic mice

Two transgenic mouse lines were created, one expressing IgM light chain genes, the other IgM heavy chain genes from an anti-murine erythrocyte autoantibody derived from hybridoma 4C8. Both transgenes in these mice were expressed in the spleen, lymph node, bone marrow and thymus [40].

Mating the above mice produced double transgenic mice with antibody specific for murine red blood cells (RBC's). These mice had three different phenotypes: 51% of the mice were tolerant, 41% were intermediate whilst others, around 5%, were anaemic. It was also found that tolerant mice can become anaemic and vice versa.

Further investigation showed that lymph node and splenic expression of the anti-RBC B cells in tolerant mice was greatly reduced. This was not seen in the bone marrow, suggesting deletion of auto-reactive B cell clones, probably at the pre-B cell to B cell transition on encountering antigen within the bone marrow. Serum auto-antibody was also reduced in tolerant mice [41] [42].

d) Anti-CD8 immunoglobulin transgenic mice

The CD8 α chain gene in mice has two alleles, CD8.1 and CD8.2. The CD8.2 allele is recognised by a monoclonal antibody, the V region genes of which, plus either the Cμ membrane (m) and secretory (s) or Cμ membrane only, were cloned into the germline of mice producing two transgenic lines [43]: the anti-CD8.2μ(m+s) and the anti-CD8.2μ(s) respectively.

Backcrossing the former transgenic mice onto a mouse background positive for the CD8.2 antigen resulted in tolerance as seen by the lack of detectable anti-CD8.2 antibody. In contrast, crossing the anti-CD8.2μ(s) transgenic mice onto this background did not result in tolerance and anti-CD8.2 antibodies could be detected.
After lipopolysaccharide (LPS) stimulation in vivo spleen cells from anti-CD8.2μ(s) but not anti-CD8.2μ(m+s) transgenic mice resulted in production of anti-CD8.2 antibodies.
On a CD8.1 background both sets of transgenic mice made anti-CD8.2 antibodies and the mice were not tolerant. The authors of this work suggested that in these mice tolerance was due to deletion of self-reactive cells since previous experiments had suggested that a) anergic B cells can be rescued in hybridomas and b) that deletion occurred for multivalent membrane antigens.

e) IgD transgenic mice
The role of IgD in tolerance was investigated in mice that lacked IgD [44]. These mice had 30-50% fewer B cells in the spleen and lymph nodes however there was a normal number of pre-B cells which expressed 2-3 times as much IgM as control mice. These mice were capable of T independent as well as T dependent responses.
Mice were created with transgenic mIgM or transgenic mIgM and mIgD specific for the hapten trinitrophenyl (TNP). When IgD-ve anti-TNP transgenic Ig+ve mice were given high doses of TNP coupled to Bovine serum albumin (BSA) deletion of mature bone marrow B cells occurred at a high frequency. However for those mice expressing both IgM and IgD, tolerance was abrograted: thus the presence of IgD may have protected mature B cells from deletion such that they could generate immune responses.
These mice show that a negative signal via the mIgM receptor on B cells results in deletion of autoreactive B cells when in contact with the auto-antigen and that mIgD does not induce additional negative signals but may modulate this signal [45].

f) Anti double stranded (ds) DNA transgenic mice.

Deletion of B cells with specificity for dsDNA has also been shown [26]. In transgenic mice expressing light and heavy chains which encode an antibody ($V_H3H9/V\kappa4$) [46] with an idiotype specific for single stranded (ss) and dsDNA, B cells with dsDNA specificity were undetectable. In another transgenic mouse expressing anti-ssDNA idiotypic antibody ($V_H3H9/V\kappa8$) no deletion of these B cells occurred. By mutating the CDR2 region of the V_H3H9 transgene, the resulting transgenic heavy chain, V_H56R, when combined with endogenous light chains $V\kappa4$, $V\kappa8$ or $\lambda1$, has high affinity for both ss and dsDNA.

Analysis of the $V_H56R/V\kappa8$ transgenic mice showed that unlike the $V3H9/V\kappa8$ tg mice the frequency of B cells, as shown via FACS analysis, was about 40% of that of the non-transgenic littermates. These mice were not immunodeficient since they had normal frequencies of T cells. V_H56R only tg mice, which bound to an endogenous light chain which conferred dsDNA specificity, also had 40-50% fewer B cells than V_H3H9 only tg and non-transgenic mice.

Antibody production in these mice was analysed using hybridomas created from LPS stimulated tg splenic cells. Testing the hybridomas for anti-dsDNA antibodies revealed that autoantibody production was severely reduced, 1/63 clones (both tg mice) produced dsDNA which might have been the result of this hybridoma expressing both endogenous and transgenic H chains. In conclusion deletion of B cells specific for membrane bound dsDNA can occur.

ii) Silencing/functional arrest

a) HEL and anti-HEL single transgenic mice

Transgenic mice expressing HEL under the control of the zinc inducible metallothionein promoter [47] were created. Unlike the previously mentioned HEL-transgenic (HEL-tg) mice these mice expressed the HEL protein as a soluble protein and lines of mice expressed either high or low levels of this protein.

When one of the high HEL expressing lines, line ML5 (13-20ng/ml-1), was immunised with HEL plus complete freunds adjuvant (CFA) no anti-HEL antibodies and no IgM nor IgG plaque forming cell (PFC) were detected. Non-transgenic mice made high antibody titres to this protein and IgG PFC were present.

In these transgenic mice it was shown that although T cells were also tolerant in vitro and in vivo the inability to respond at the antibody level was due to B cell tolerance. When the ML5 tg line were injected with HEL coupled to SRBC, which provides an alternative T cell help, these mice produced an anti-SRBC response although the anti-HEL response was decreased by 50 fold.

Anti-HEL tg mice were created expressing high affinity heavy (H) and light chain V regions with an IgM+D+ constant region [47]. The heavy chain of this antibody expressed a BALB/c IgH^a allotype marker which allowed the distinction between endogenous H chains, IgH^b allotype, and the transgenic chain. In these mice 90% of the splenic B cell expressed the tg IgM. Whilst only 1-6% of these cells possessed endogenous antibody a large number of plasma cells from these mice were found to express endogenous IgM [48]. B cell differentiation in these mice was found to be the same as non-transgenic mice, with development occurring normally at the bone marrow pre-B to immature B cell stage, although the number of pre-B cells was reduced. B cells also located to the correct microenvironments giving rise to well developed follicular mantle zone as well as splenic marginal zones [48].

Immunisation of these mice with HEL plus CFA resulted in the production of high anti-HEL IgM titres.

b) HEL X anti-HEL mice, the double transgenics.

These Dbl-tg were progeny of a cross between mice transgenic for the soluble HEL gene (line ML5 high lysozyme expressing cells) and mice transgenic for the heavy and light chain genes encoding a high affinity anti-HEL antibody [49].

Testing for HEL tolerance in 6 week old Dbl-tg revealed that these mice were tolerant to HEL at both the antibody and PFC levels. This was also shown when anti-HEL tg bone marrow was transferred into ML5 HEL tg mice and following immunisation of iradiated C57BL/6 mice reconstituted with Dbl-tg spleen cells and C57BL/6 T cells primed with HEL-HRBC (horse red blood cells). Thus Dbl-tg mice are tolerant to HEL even in the presence of T cell help.

These Dbl-tg mice were tolerant inspite of the presence of anti-HEL+ve B cells. This suggested that deletion of autoreactive B cells in these mice was not the mechanism of tolerance induction (cf membrane bound HEL above) but the result of functional silencing (anergy) of autoreactive B cells. These anergic B cells had down regulated membrane IgM levels but the expression of IgD, B220, J11D, Ia, Ly-1 and Mac-1 remained unaffected. The down regulation of IgM on tolerant B cells was not due to non-association of IgM heavy chains with mb-1 and B29, which form the heterodimer associated with membrane expression of immunoglobulins. IgD on tolerant B cells was also associated with these molecules. It was shown that IgM heavy chains were associated with mb-1 and B29 but this complex appeared not to exit from the endoplasmic reticulum, and no Golgi processing was evident [50].

8 week old Dbl-tg mice also displayed these characteristics with bone marrow and lymph node cells of these mice having similar changes. It was however noticed that in these mice there was a decline in the number and frequency of B cells, which may have been due to deletion of these cells.

The development of the B cells in these mice was normal until they reached the follicular mantle zone stage. Splenic marginal zone and red pulp were both devoid of transgenic B cells [48]. It has recently been shown that the life span of these anergic B cells, once they reach the periphery, is greatly reduced and unaffected by the expression of HEL [51].

Double transgenic mice expressing low level of lysozyme (line ML3) were also created. These mice had no reduction in the serum levels of anti-HEL IgM antibody as well as no reduction

in B cell numbers as compared to non-transgenic mice. In these Dbl-tg mice self-reactive T cells were absent [52].

Expression of the HEL-transgene was induced in these transgenic mice following zinc feeding (in drinking water) for 1-4 days. Increased HEL secretion corresponded with a decrease in IgM levels on the B cells although there was no reduction in the number of B cells in these mice [49]. Adelstein et al (1991)[52] also showed that giving zinc to another low expressing HEL-transgenic mouse line, ML4, resulted in increased serum HEL levels and a marked decrease in both the number and affinity of responding lysozyme specific B cells, as compared to non-transgenic mice.

In these HEL induced transgenic mice it was shown that both immature B cell and mature B cells were tolerised. Immature B cells could have been tolerised at the level of the bone marrow since staining with anti-HEL antibody revealed that immature $B220^{lo}$ B cells had bound HEL and around 50% of receptors were occupied. These immature B cells also had down regulated mIgM levels [48]. Tolerance to HEL was induced in non-transgenic mice when bone marrow from Dbl-tg mice was transferred with T cell help and antigen.

Tolerance of B cells also needs to occur in the mature B cell population since, unlike the T cell receptor, mature B cell receptors undergo further diversification through somatic hypermutation. The resulting cells may express high affinity self receptors.

Experiments to test that mature self-reactive B cells can be effectively tolerised involved the investigation of low-lysozyme expressing (ML3) Dbl-tg mice [49]. Irradiated non-transgenic mice were primed with HRBCs before being given anti-HEL Ig (Ig-T), zinc uninduced or zinc induced HEL low expressing Dbl-tg splenic B cells. Following immunisation with HEL-HRBC, recipient mice given Ig-T and the zinc uninduced Dbl-tg spleen cells respond to HEL in contrast to those receiving zinc induced Dbl-tg B cells which responded poorly. Thus increasing the levels of HEL resulted in tolerance of the mature B cell population. If primed T cells were also

transferred, the mice remained unresponsive. Unresponsiveness was not due to receptor blocking by HEL since transferring Ig-T only B cells which had half of their receptors blocked in vitro did not result in tolerance.

Mature B cell tolerance was also shown by transferring Ig-T only or non-transgenic splenic B cells to irradiated HEL-transgenic mice expressing high levels of HEL or to irradiated non-transgenic controls. HEL expressing transgenic mice remained tolerant and the level of mIgM on transferred cells decrease by ten fold. Thus mature Ig-T B cells could be tolerised to soluble HEL. This unresponsiveness was not reversed by the addition of helper T cells.

Having established that tolerance to HEL occurs in both the immature and mature B cell populations of mice expressing sufficient levels of HEL, the question arose as to whether this was an intrinsic property of the B cells themselves. Through the use of irradiation chimaeras it was shown that tolerance was due to an intrinsic functional change in the anergic B cells themselves; transferring Dbl-tg but not Ig-T spleen cells into non-trangenic mice resulted in an animal unresponsive to HEL. This was not attributable to the presence of suppressor cells transferred in the spleen mixtures [53]. B cells from Dbl-tg mice also responded poorly to HEL as well as to non-specific stimuli (LPS) in vitro as compared to Ig-T only mice. However after stimulation with LPS for 7 days they responded to the same extent as control cells. This suggested that a reversal in tolerance could occur in these anergic B cells. This was further shown by Goodnow et al (1991) [54]. Transferring B cells from the HEL-tolerant Dbl-tg mice plus helper T cells primed to RBC into non-transgenic or HEL (expressing tolerogenic levels of HEL) transgenic mice, resulted in non-transgenic mice capable of responding to HEL-RBC whilst HEL-only recipients remained unresponsive. Although the reversal of tolerance occurred once B cells were removed from an HEL environment, the level of antibody responses to HEL was never equivalent to that of Ig-T mice. "Parking" Dbl-tg cells within a non-transgenic mouse for a period of 10 days resulted in

spontaneous mIgM recovery on tolerant cells, but not spontaneous anti-HEL antibody production. This was not due to expansion of new pre-B cells. An anti-HEL response only occurred following a secondary boost of HEL antigen suggesting that antibody production reversiblity may require many rounds of mitogenic stimulation either with T_H or LPS in the absence of HEL antigen.

These anergic B cells have been shown to constitutively express HEL. However this has not yet been found in HEL expressing transgenic mice [55, 56]. Dbl-tg anergic B cells have been shown to constantly stimulate HEL-specific T cell hybridomas and they can present a specific HEL peptide, HEL_{46-61}. This expression of HEL is not due to synthesis of the protein by B cells themselves but through the binding of HEL present in the Dbl-tg mice. Reconstituting an irradiated HEL expressing Dbl-tg mouse with Ig-T bone marrow (B cell are not capable of producing HEL) resulted in B cells which could stimulate T cell hybridomas. These cells could also stimulate HEL-specific T cells from immunised mice.

Although B cells from Dbl-tg mice were capable of stimulating T cells this was not as effective as Ig-T B cells plus exogenous HEL. Addition of HEL to Dbl-tg B cell did not increase their ability to stimulate T cell hybridomas suggesting that the Ig-receptor dependent uptake of antigen and antigen presentation is impaired. This maybe due to the reduced level of mIgM on anergic cells since mIgD can process and present HEL. Tolerant B cells from the Dbl-tg mice were unable to trigger an increase in intracellular tyrosine phosphorylation, Ca^{2+} release or subsequent B cell activation following binding of HEL to membrane Ig.

It was also noticed that anergic Dbl-tg B cells had no defect on their ability to process and present nominal antigens such as keyhole limpet haemocyanin (KLH).

Anergic B cells however respond to other signals which mediate later B cell activation responses. Stimulating Dbl-tg cells <u>in vitro</u> with IL-4 and anti-CD40 resulted in cells with the capacity to respond to T cell help. Signalling occurred in

Dbl-tg mice using the membrane form of HEL mIg and this also restored their B cells' ability to collaborate with T_H cells and resulted in antibody production [57]. The role of CD40 was also investigated by Eris et al (1994) [55]. T_H cell membranes expressing the CD40 ligand stimulated B cells from Dbl-tg mice resulting in the upregulation of mIgM. In the presence of both activated T_H membranes and cytokines, anergic B cells produced anti-HEL antibodies. This was inhibited if a soluble CD40-Ig fusion protein was added to the cultures. Anergic B cells from Dbl-tg mice plus HEL-primed T cells plus HEL in CFA injected into an irradiated Dbl-tg mouse resulted in mice which, when stimulated with HEL made an anti-HEL response equivalent to Ig-T mice. This may have been due to CFA increasing receptors such as the CD28 ligands on B cells (these are expressed at reduced levels in anergic B cells), thus increasing T cell help to B cells.

c) Anti-DNA immunoglobulin transgenic mice

As described above, anti-DNA tg mice were created expressing anti-DNA specific V region genes from a hybridoma derived from a MRL-lpr/lpr mouse, joined to an IgM constant region but not the IgD region. The V_H3H9 gene can combine to any endogenous light chain to give anti-ds or ssDNA specific antibodies. Mating V_H3H9 tg mice with a $V_\kappa8$ transgenic resulted in mice specific for ssDNA. These tg mice had a high frequency of anti-DNA B cells which bound DNA and anti-DNA serum titres were not greater than normal mice, which have low serum anti-DNA IgM levels.

The V_H3H9 only transgenic also confirmed these data. 38-86% of these tg mice splenic B cells were IgM+ve and bound ssDNA; the rest did not bind DNA. Looking at hybridomas created from splenic B cells it was found that although 52% of hybrids secreted ssDNA antibodies the levels of serum anti-ssDNA never exceeded that found in normal mice. Therefore it appears that tolerance to ssDNA occurs through functional silencing, i.e. although these cells are present they do not differentiate into antibody forming cells. This functional

silencing appears to be due to a signalling defect in the B cell since expression of high levels of mIgM did not inhibit anergy [46].

Tolerance to DNA was also studied by Tsao et al (1993) [58]. They created an IgM transgenic mouse expressing the VκJκ regions of an IgG2a monoclonal antibody. It had been shown previously that tg mice encoding the IgG2a monoclonal antibody were not tolerant and this resulted in mice developing a non-fatal autoimmune condition. These IgM tg mice expressed the transgene at high levels on the surface of most splenic B cells. As compared to non-transgenic mice there appeared to be no reduction in the number of B cells in the tg mice and they did not secrete transgenic IgM antibodies. These mice appeared to be tolerant through functional silencing since this tolerant state could be over come by in vitro incubation of tg IgM spleen cells plus LPS, which resulted in an increase in secreted IgM anti-DNA antibody.

Anergy to dsDNA has also been shown [59]. Non-autoimmune tg mice expressing anti dsDNA antibody had reduced spleens (14-39% of the non-transgenic mice) and they also had 50-70% fewer B cells. This was due to the loss of B cells, suggesting a deletional tolerance to dsDNA. However the remaining B cells had an immature phenotype and were shown to be anergic dsDNA specific B cells. These cells however could be stimulated to produce antibody specific for dsDNA with LPS over a period of a few days in culture. In vivo these tolerant cells produced little or no dsDNA antibody. The antibody produced was non-pathogenic.

d) VSV-GP transgenic mice.

Steinhorff et al (1993) [60] created two transgenic lines expressing the membrane glycoprotein (GP) of vesicular stomatitis virus (VSV) under the control of two promoters: SV40 (line 207) and myelin basic protein (MBP) (line 163). Transgene expression was found in the kidney, heart, thymus, lung, brain, liver, and central nervous system.

Immunisation of these transgenic lines with recombinant vaccinia virus expressing VSV-GP resulted in no measurable IgG immune response. However if these mice were immunised with either wild type VSV or VSV-GP covalently coupled to sperm whale myoglobulin, a strong immune response resulted with the production of high titres of high affinity neutralising IgG antibodies. These data suggested that although the tg mice were tolerant to VSV-GP, probably through anergy, the tolerance could be broken by different forms of the antigen. This ablation of tolerance was T cell dependent: removal of CD4+ve T cells resulted in no VSV-GP auto-antibodies.
The influence of antigen organisation on B cell tolerance was also shown by Bachmann et al (1993) [61]. Transgenic mice were created that expressed VSV-GP (serotype Indiana (IND)) under the control of the H-2K^b promoter. When these mice were immunised with "poorly" organised antigen, soluble VSV-GP (IND) or 2x10^6 PFU recombinant virus expressing VSV-GP (IND) cells no measurable IgM or IgG response was evident. Control mice responded well to these antigens. Thus these transgenic mice were tolerant to the product of their transgene. The tolerance was transferable to non-transgenic mice, but tolerance was broken when these mice were immunised with a "highly" organised form of the antigen which results in high titres of neutralising antibody. Thus it appears that although tolerance had occurred cells remained which had the potential to respond to other forms of the antigen, in particular repetitive arrangements of the antigen. It should also be noted that in these VSV-GP tg mice the antigen is membrane bound, thus anergy can perhaps occur to membrane proteins which are at a low density.

iii) B cell tolerance summary

The presence of a self protein either membrane bound or in a soluble form can induce B cell tolerance, in the presence or absence of T cell tolerance. Two mechanisms of B cell tolerance have been described; 1) deletion and 2) anergy. Deletion of self-reactive B cells occurs in the bone marrow at

the pre-B cell to immature B cell transitional stage following mIgM interaction with a membrane bound protein. This process occurs in two stages, firstly arrested development and secondly cell death.
Anergy occurs following mIgM interaction with soluble antigen (although anergy has been shown to occur upon contact with membrane bound antigens) and both immature and mature B cells can be tolerised in this way. This process is thought to be the result of biochemical changes affecting the B cell receptor complex and not signals that mediate later B cell activation processes.

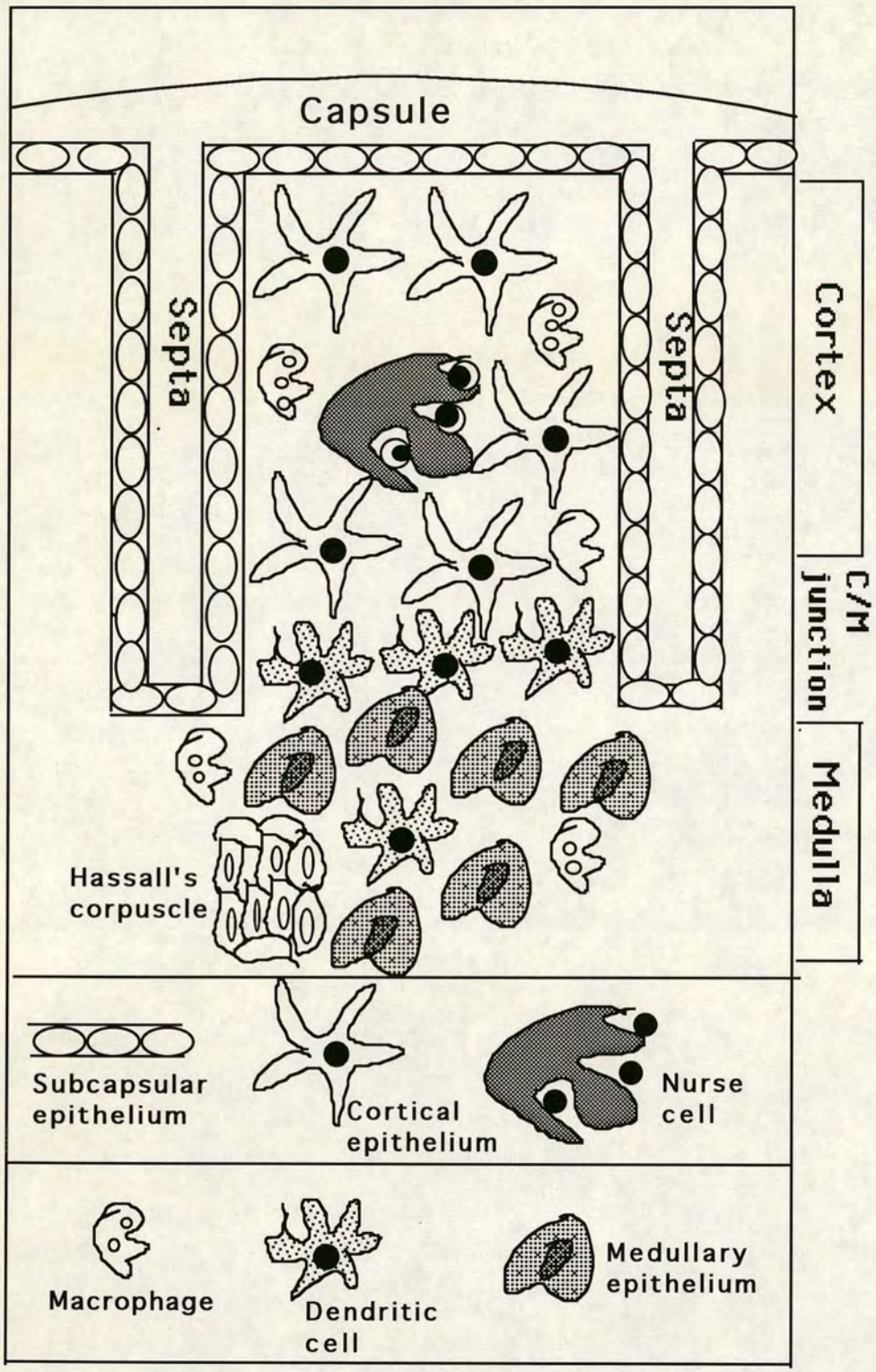
1.3) Thymic architecture and T cell differentiation

i) The thymus

a) Introduction

The organ in which T cell differentiation occurs is the thymus. Within the thymus T cells mature and proliferate, they rearrange and express the T cell receptor (TCR), and they undergo two selection procedures; positive and negative selection. T cells emerging from the thymus express a TCR capable of recognising foreign antigen in the context of self MHC. Apart from TCR expression T cells express different surface molecules throughout development. Two of these, CD4 and CD8, have been implicated in influencing the specificity of the maturing T cell.
Many reviews have described the structure of the thymus, see [62-68]. The following section presents a brief summary of work described in these reviews.
The thymus is a bilobed structure that lies just above the heart in adult mammals. Surrounding each lobe is a capsule of connective tissue that indents periodically to create septa which further divide the lobe into lobules. Within these septa are the vascular and neural supplies to and from the thymus and the wide blunt ends of the septae form the perivascular space. In this area exchange of both cells and solutes between the perithymic stroma and the periphery occurs. It is also rich

Fig. 1.3. Schematic diagram of a thymuc lobule



Schematic representation of the stromal cell population within an individual thymic lobule. All intervening spaces are filled with thymocytes (not shown).
Diagram modified from [65].

in various cells including plasma cells, mast cells, myeloid cells and thymic B cells, either scattered individually or in lymphoid follicle or germinal centres. A basement membrane is found below the capsular region and this is also found around thymic blood vessels.

Each lobule is made up of various regions, starting from the outside and moving inward, the subcapsular, the cortex, the cortico/medullary (CM) junction and medulla regions (see Fig. 1.3). Each of these areas are composed of histologically distinct epithelial cells.

Within the murine thymus, epithelial cells within these regions do not represent a homogenous population, (heterogenity being indicated by keratin staining, light and electron microscopy (EM) (Type 1-6) and monoclonal antibodies (CTES 1-5)). Heterogenity was also found at the MHC level for both Class 1 and Class 2. Although all thymic epithelia express MHC Class1 at high levels MHC Class 2 expression is not as simple. MHC Class 2 is present at high levels on all cortical epithelia whilst medullary cells also possess Class 2, but the level of expression differs between species. For example, in mice a large proportion of these cells are Class 2 positive. Subcapsular epithelial cells are negative for Class 2.

Bone marrow derived cells are also found throughout the thymus. Monocyte and dendritic cell precursors enter the thymus where they differentiate into macrophages and interdigitating cells respectively. Macrophages are present in the cortex and CM junction. It is thought that these macrophages are involved in the removal of dead cells as well as produce cytokines which are important in T cell differentiation. Since these cells also express MHC Class 2 they may act as antigen presenting cells. Interdigitating cells are found in the medulla and CM junction. These cells express large amounts of MHC Class 2 and may therefore act as antigen presenting cells. Mature T lymphocytes are also present in the medulla. Bone marrow derived cells have been implicated in both positive and negative selection events (see later sections).

b) Thymic epithelia and T cell differentiation

The importance of the thymic environment is evident when the thymus is observed following sublethal irradiation [69, 70]. A close relationship exists between the stromal cell architecture of the thymus after irradiation and reappearance of lymphoid cells that repopulate the thymus during the first phase of regeneration. Following whole body irradiation the cortex is reduced in size and is largely depleted of lymphoid cells as well as MHC Class 2 expression. In contrast the medulla is less affected. After 5 days the thymic cortex stroma is restored and at this time MHC expression reappears, coinciding with the vascularisation and repopulation of the cortex by T cell progenitors.

It has been suggested that haematopoietic precursors cells migrate to the thymus by chemotaxis, the directional migration of cells along soluble gradients of chemical substances called chemoattractants. Chemotactic factors are thought to be produced by the thymus itself. Rat subcapsular and perivascular epithelia (Type 1) cells produce and secrete an 11kDa protein that selectively attracts immature lymphoid cells in vitro. This protein was named Thymotaxin [71, 72]. However recent experiments have shown that this molecule is β_2m [73], which is associated with the α3 domain of MHC Class 1 as well as the non-classical MHC molecule CD1, both of which are found on thymic epithelia (CD1 found on cortical cells only). β_2m from mouse and human epithelial cells has also been shown to be a chemoattractant.

Although β_2m appears to be important for thymic homing it is not the sole chemotactic factor since T cell migration occurs in mice lacking the β_2m gene. Subcapsular and medullary epithelial cells produce polypeptides factors termed thymic "hormones". These hormones were indentified from crude thymic extracts and have been shown to be biologically active pre-thymically and in the periphery. Two main properties have been described. Firstly they are involved in the phenotypic

maturation of bone marrow progenitors and secondly in the modulation of mature T cell functions.

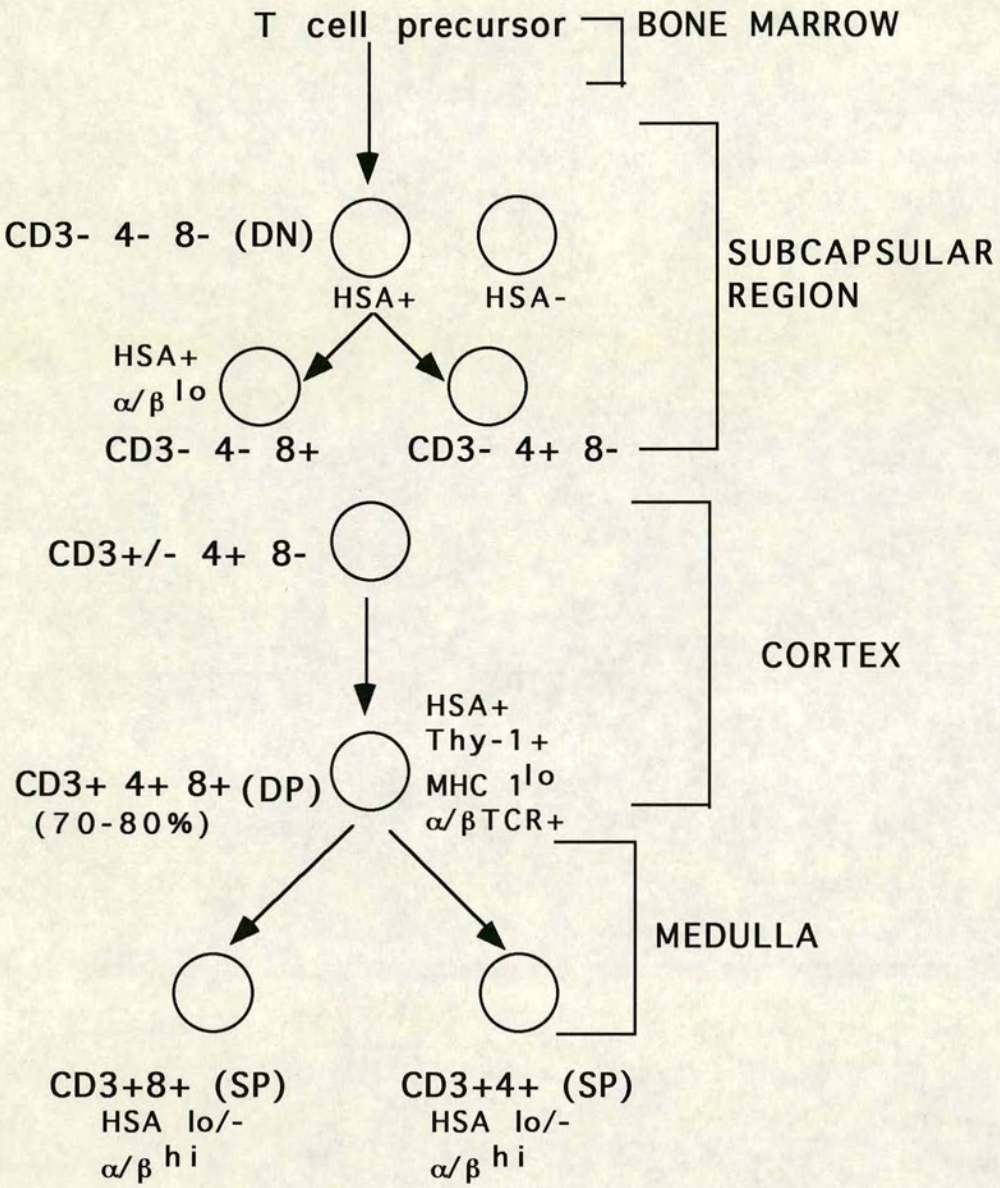
If murine bone marrow progenitor cells are separated, using an albumen gradient, and stimulated with these thymic hormones a rapid increase in Thy-1, CD5, CD8 and Tdt expression occurs. This suggests that thymic hormones caused the expression of an already synthesised intracellular pool, rather than causing de novo synthesis. Whether they affect intra-thymic development is still questionable however the fact that thymic hormones are produced by epithelial cells that first contact thymocytes progenitor cells suggests that they may be important at the early stages of T cell development.

Within the cortex some Type 2/3 epithelial cell are found completely engulfing thymocytes. These cells are known as thymic nurse cells (TNC) [74-79] TNC's are large structures (50μm in diameter) expressing MHC Class 1 and 2 but lacking sIg and Thy-1 and have been isolated, following intensive trypsination, from chicken, sheep, mouse, rat and human thymic tissue. These structures are also observed early in fetal development, around day 17 in mice which is an important time in T cell ontogeny.

Murine TNC's have been shown to contain 20-40 small to medium sized mitotic T cells although this number can vary between 10-200 (in man 1-2 up to 20-30). EM analysis has shown in vivo that small gaps exist in the surrounding TNC membrane such that lymphocytes can enter and leave this structure. T cells found within these complexes have a distinctively immature phenotype, CD4+8+, and are sensitive to cortisone and radiation. Removal and stimulation (with either alloantigens, mitogens and growth factors) results in intra-TNC T cell proliferation and IL-2 production. Thymocytes released from TNC's have been shown to express TCR at a higher density than other cortical T cells and in vitro these T cells are incapable of forming other associations with thymic epithelia.

TNC and T cell interactions maybe due to receptor-mediated recognition of epithelial cells by thymocytes in a random

Fig. 1.4. T Cell differentiation



interaction, in as much as most cortical T cells express the receptor but only those coming in close proximity to TNC's bind. Monoclonal antibodies to CD8, CD4, MHC Class 1 and 2 interfere with these interactions, with anti-CD4 and anti-MHC Class 1 or anti-CD8 and anti-MHC Class 2 combinations completely inhibiting TNC formation.

Since TNC are closely associated with CD4+8+ T cells it has been suggested that they are involved in either positive or negative selection events, which shape the T cell repertoire, with most evidence implicating their involvement in positive selection events. Despite this, TNC tested in vitro for their antigen presenting properties were incapable of presenting antigen to Class 2 restricted T_H cells. This maybe due to a lack of processing ability however they maybe capable of presenting previously processed peptides or endogenously synthesised membrane proteins. Even if they play no part in the selection events TNC epithelial cells produce a thymic hormone named Serum Thymic Factor which might affect T cell development.

In the medullary region Type 6 epithelial cells form the Hassall's corpuscles (tubular clusters of epithelial cells). The function of these is unknown, however it is thought that they may act as a thymocyte "graveyard", since they are associated with cell debris and products of cell death.

Epithelial cells are also involved in positive and negative selection events and their roles in each of these will be discussed in later sections.

ii) Intra-thymic development.

a) Thymocyte populations as described by surface markers, CD4 and CD8 (see Fig. 1.4).

Committed bone marrow stem cells enter the thymus and these prothymocytes divide, mature phenotypically and functionally, whilst undergoing the already mentioned selection events. As they mature phenotypic changes occur which will be described in this section.

Early T cell progenitors, in mice, enter the thymic rudiment through the capsule and once the thymus has been vascularised they enter via high endothelial venules in the CM junction before moving to the subcapsular region. The nature of the bone marrow cells seeding the thymus is unknown, however Ardavin et al (1993)[80] isolated early pro-T cell precursors. These cells resembled bone marrow haematopoietic stem cells except they also expressed Sca-2 and low levels of CD4. These cells also express TCR genes in the germline configuration and $\alpha\beta$ and $\gamma\delta$ positive T cells have been seen following intrathymic injection of these cells. It has also been observed that these progenitor cells can give rise to B cells as well as dendritic cells following intravenous and intrathymic injection respectively.

Early T cell progenitors in the thymus give rise to the immature CD4-8-, the double negative (DN) T cells population. These cells make up 1-2% of the thymic population. This population of cells is heterogenous as shown by the expression of the heat shock antigen (HSA). HSA+ cells have precursor activity in that intravenous or intrathymic injection of these DN cells results in the repopulation of the thymus. HSA-ve DN are devoid of this property. DN cells have increased IL-2Rα and Thy-1 expression but a decreased Pgp-1 levels.

It is at this stage that immature thymocytes rearrange their TCR genes in the order of γ, δ, β and α. The β TCR chain has been detected on the surface of DN cells in TCRβ transgenic mice as well as on the surface of SCID thymus pre-T cell lines transfected with a TCRβ gene. This TCRβ protein is found in association with a glycoprotein, gp33 and CD3ε. This is not a transgenic artefact since 20% of thymocytes from a 16 day old normal mouse embryo also express low levels of TCRβ and gp33.

DN differentiate further, some upregulate CD4 or CD8 to become immature single positive cells which either express some CD3 or none at all. Regardless of the route taken these immature single postive cells become double positive cells (DP) CD4+8+ after passing through a CD4+/-8-3+ stage. At this

stage of development there is an expansion of cells and the DP population make up 85-90% of the thymocyte population.
This is also not a homogenous population of cells. In the mouse 25% of the DP cells are large dividing cells whilst the rest, 75%, are small dividing cells. These cells express intermediate levels of CD3 as well as the αβ TCR. Transition from DN to DP has been shown to be dependent on the expression of the β chain of the TCR. Transgenic mice lacking the β chain have T cell developmental arrest at the DN stage whilst mice lacking the α chain contain DP cells [81-84].
DP cells do not have progenitor activity nor are they functionally mature.
This stage in T cell development has been implicated as the stage in which thymic selection processes are thought to occur since it coincides with an enormous loss of cells through apoptosis. About 97% of DP cells die. As mentioned above positive selection results in a population of thymocytes specific for self MHC whilst negative selection results in the removal of thymocytes that recognise self MHC plus self peptide with high affinity. The resulting population of T cells are the single positive (SP) cells either capable of recognising foreign antigen in association with either MHC Class 1, (CD8+4-3+) or MHC Class 2, (CD8-4+3+). These cells express high levels of αβ TCR and they have aquired some of the properties of mature peripheral T cells [85]. Mature SP T cells leave the thymus probably via the CM junction high endothelial venules or via the lymphatics.
Kinetics of the acquisition of these cell surface markers have been described elsewhere [86].

b) Changes in location.
As desribed above fetal progenitors enter the thymus through the large venules in the medulla and at the CM junction, they then migrate to the subcapsular region. As the cells move through the subcapsular region their phenotype is DN. The main phenotype of the cortex T cells is that of the DP and it is here

or at the CM junction that selection occurs since only SP cells are present in the medulla.

c) T cell receptor expression in the thymus.

Mature peripheral T cells recognise antigen in the form of a processed peptide in the context of an MHC molecule on an antigen presenting cell via the T cell receptor (TCR). There are two types of TCR's, ∂δ and αβ the most common being the later. Both receptors recognise MHC.

The αβ TCR consists of an outer variable region, a constant region, a transmembrane region and a cytoplasmic tail. It is the variable region that recognises specific antigen plus MHC. This region is a product of somatic rearrangement of both α loci V and J elements and the β loci V,D and J elements via a recombinase system, Tdt and RAG 1 and 2.

In mice the β TCR loci is found on chromosome 6 and contains 20-25 V elements which are separated from several constant regions by J and D regions. During rearrangements of these genes one D is juxtaposed to one J, and this DJ product is then combined to one of the variable elements. It has recently been shown that the rearrangement of the TCR β loci is not random, certain V regions combine with certain J segments.

The β chain rearrangement occurs after the ∂δ TCR gene rearrangements and a decrease in the number of ∂δ TCR+ve cells in the murine coincides with the development of αβ TCR. The rearrangement and expression of the β chain protein results in allelic exclusion of further β chain rearrangements. Observations in transgenic mice in which a functionally rearranged TCR β gene was added suggested that the presence of the rearranged β gene product resulted in the inhibition of endogenous β TCR gene rearrangement. This "allelic exclusion" is dependent on expression of the TCR β protein, since transgenic mice expressing only full length RNA rather than protein could not suppress rearrangement.

Mice in which both Vβ2 and Vβ8.2 transgenes were introduced expressed both receptors on the surface of mature peripheral T cells. These data suggest that allelic exclusion must occur at

the DNA rearrangement level to prevent co-expression of two distinct TCR genes [87].

The β chain of the TCR was shown also to be important for clonal expansion of immature thymocytes. Transgenic mice expressing a non-functional β gene (with a deletion in the variable region which inhibits rearrangement) have normal numbers of thymocytes with the CD4+8+ phenotype. Maturation, however, does not proceed past this point unless a functional β chain was introduced [88].

A further role for the TCR β chain was shown using transgenic mice lacking this chain. T cell development is arrested at the DN stage. In transgenic mice lacking the α chain genes, but which express the β TCR chain, have DP cells thus expression of the β chain is important for the transition from DN to DP [81-83].

Mice lacking recombinase activity, SCID mice and RAG 1 and 2 deficient transgenic mice have T cell development arrested at the DN stage. Introduction of a rearranged TCR β gene into these mice and β TCR-ve mice results in the transition from DN to DP cell type. Therefore TCR β chain expression may induce CD4 and CD8 expression [81, 84].

The TCR α locus also undergoes recombinational events. In mice the TCR α and TCR δ genes are both found on chromosome 14, the δ element found within the α loci. TCR α chain rearrangement occurs after the β chain. There are no D segments in the α loci and the recombination event is only a V-J rearrangement. These events are non-random.

The TCR α chain is independent of the TCR β chain as shown in β knockout mice. In these mice thymocytes express TCR α [81]. Transgenic mice lacking TCR α have thymocyte numbers similar to non-transgenic littermates. They possess normal levels of DP cells but lack CD4+ and CD8+ single positive cells and a thymic medullary region suggesting that the role of the TCR α is for further differentiation to single positive thymocytes [81, 83].

TCR α rearrangement is not influenced by allelic exclusion and TCR α rearrangements occur until the time of positive

selection and RAG 1 and 2 gene switchoff. This would increase the available receptor repertoires [89]. TCR α chain expression does not affect $\partial\delta$ thymocyte production [90]. TCR expression kinetics have been described elsewhere [91, 92].

1.4) Positive selection

Positive selection is the event during which developing T cells become MHC restricted as well as becoming committed to either the CD4 or CD8 single positive cell lineages recognising MHC Class 2 or Class 1 respectively. Positive selection is influenced by the MHC molecule expressed by the thymus as well as the peptide bound to it. The specificity of the T cell receptor (TCR) and the co-receptors CD4 and CD8 have all been implicated in positive selection.

i) MHC restriction and the importance of the TCR-MHC interaction in this process.

Early bone marrow experiments suggested that T cells developing within a thymus became restricted to the MHC expressed in this organ. For example bone marrow from a F1 (axb) MHC haplotype offspring into a parental host with MHC haplotype 'a' resulted in T cells which recognised foreign antigen in association with 'a' MHC haplotype [93]. The mechanism of MHC restriction is now thought to depend on positive selection in the thymus.

Transgenic mice have further confirmed the existence of MHC restriction and also indicated that the TCR specificity of the developing T cells was important [94-102].

These papers have provided evidence for the involvement of the TCR in MHC restriction. In general the experiments involved the use of mice expressing transgenic (tg) TCR$\alpha\beta$ receptors (2C tg TCR, [94]; H-Y specific tg TCR, [95, 97]; lymphocytic choriomeningitis virus (LCMV) tg TCR, [102] and pigeon cytochrome c tg TCR [101, 103, 104] specific for antigen (L^d Class 1, male H-Y antigen, LCMV and pigeon cytochrome c, respectively) in the context of a specific MHC Class 1 or 2 molecule. The above tg TCR's are positively

selected in mice expressing the following selecting MHC molecule; H-2K^b, H-2D^b, H-2D^b or I-E^k respectively, and the resulting cells are CD4-8+ transgenic TCR+ve (high expression) for Class 1 or CD4+8- for MHC Class 2. On a non-selecting background these cells are not present.

Bone marrow transfer studies also confirmed the importance of MHC interactions for positive selection [95, 97]. Reconstitution of female H-2D^b (selecting MHC) but not H-2D^k thymus (non-selecting MHC) with bone marrow cells derived from αβ H-Y tg TCR mice resulted in the elevated production of CD8+ve cells expressing the transgenic TCR [95]. A similar experiment was also performed by Kisielow et al (1988) [97] and the same results observed. No positive selection was observed on non-selecting MHC alleles K^k, D^k, K^d, D^d or K^b. The level of tg TCR on DP cells was high regardless of MHC background. (In these experiments female mice were used since they did not express the male H-Y antigen which has been shown to induce tolerance; see negative selection below).

The DP stage in thymocyte development is the stage at which thymocyte differentiation is halted on a non-selecting MHC environment [100, 104]. Berg et al (1989) [100, 104] showed that this block in development could be reversed if the selecting MHC ligand was placed on cortical epithelial cells. In this model it would appear that positive selection occurred late in development since transgenic pigeon cytochrome c specific TCR cells develop to a relatively mature stage of DP in the absence of positive selection. Scott et al (1989) [98] crossed αβ TCR transgenic mice specific for H-2^b onto a SCID (H-2^d) background (this resulted in H-2$^{d/d}$ or H-2$^{b/d}$ αβ TCR tg/SCID mice). In contrast to the small number of CD4-8- thymocytes present in normal SCID mice, tg TCR/SCID mice had elevated levels of thymocytes regardless of their MHC haplotype, although CD4 and CD8 expression only occurred in tg TCR/SCID suggesting that the maturation into DP cells required TCR αβ rearrangements. Heterozygous H-2$^{b/d}$ tg TCR/SCID mice had CD4-8+ cells and no CD4+8- cells. Both cell types were missing in the H-2$^{d/d}$ mice, suggesting that

interaction of the tg TCR with H-2^b induced positive selection which resulted in CD8 single positive tg TCR+ve cells.

ii) Cells involved in positive selection

There has been much controversy over which cells induce positive selection since both thymic epithelia and thymic bone marrow derived cells have been implicated in this process.

Class 2 expression on epithelial cells and fetal mesenchyme has been shown to be important for T cell thymic development [105]. Purified cortical epithelial cells expressing Class 2 supported the development of DP and SP cells, but not DN thymocytes. However DN cells differentiated into DP $\alpha\beta$ TCR expressing cells in the presence of both epithelial and mesenchymal cells suggesting that mesenchymal cells are essential for the early stages of thymocyte development. The beneficial effect of mesenchymal cells on DN cell differentiation can be replaced by fibroblasts. Class 2 depleted thymic stromal cells did not support the development of DP cells.

The importance of epithelial cells was also shown via the introduction of epithelial cell lines into the thymus of recipient mice [106, 107]. The thymus derived epithelial cell line 2E4 [106] expresses low amounts of H-$2K^b$ and when injected intrathymically seeds both the cortex and medulla thymic regions. This cell line persists for a long period of time in the thymus. Injecting the 2E4 (H-$2K^b$) cell line into an H-2^k mouse reconstituted with bone marrow or fetal liver cells from H-$2^{b/k}$ F1 donors resulted in tolerance to H-2^b and no rejection of 2E4 cells. Therefore thymic epithelial cells can positively select T cells. This was confirmed with another thymic epithelial cell line [107].

Through the use of β_2m transgenic mice Bix and Raulet (1992) [108] also indicated the importance of radioresistant epithelial cells in positive selection. Irradiated β_2m positive mice given fetal liver from MHC matched β_2m negative donors had CD8+TCR+ve SP cells at the same frequency as mice give β_2m positive cells, suggesting that radioresistant epithelial

cells in the β2m recipient mice were capable of inducing positive selection.

Positive selection has also been shown to occur following the interaction of TCRs with MHC on fibroblasts [109]. β2m negative mice given an intrathymic injection of MHC Class 1 expressing fibroblasts had increased levels of CD8+ve SP cells compared with mice not given these cells. The Vβ repertoire of the rescued cells depended on the MHC Class 1 molecule expressed by the injected fibroblasts.

The role of fibroblasts in positive selection was analysed in TCR tg mice. The transgenic TCR expressed in these mice recognises the alloantigen H-L^d and is positively selected by H-$2K^b$. Introducing H-$2K^b$ fibroblasts intrathymically into H-$2K^k$ (non-selecting MHC) mice resulted in large numbers of CD8+ tg TCR ^{hi} cells, similar to the numbers seen on a selecting background.

Bone marrow derived cells have also been implicated in positive selection. Irradiated β2m negative mice were reconstituted with fetal liver from MHC matched β2m negative or positive mice. A 4 fold increase in the number of CD8+ve TCR+ve cells capable of cytotoxic activity occurred if mice were given bone marrow from a β2m positive donor, as compared to mice given bone marrow from β2m negative mice. The number of CD8+TCR+ve cells never equalled that of β2m positive mice suggesting that although bone marrow derived cells can induce positive selection they do so at an inefficient rate [108].

iii) The importance of the MHC molecule in positive selection.

The importance of the MHC molecule was further shown in transgenic mice in which both Class 1 [110-112] and Class 2 [113-115] were not present on the cell surface or had been altered in some way [116].

The MHC Class 1 molecule consists of α1 and α2 polymorphic domains which are important for the binding and presentation of antigen peptide to TCR and an α3 non-polymorphic domain which binds to CD8. Aldrich et al (1991) [116] created a

transgenic mouse which expressed a hybrid Class 1 molecule, expressing the $\alpha 1$ and $\alpha 2$ domains from L^d MHC whilst the $\alpha 3$ domain was from Q7b. This molecule binds to L^d restricted peptides but does not interact with CD8 dependent CTLs. In these mice positive selection of virus-specific L^d CD8+ve CTL's does not occur. However in mice expressing normal L^d MHC positive selection did occur suggesting that the $\alpha 3$ domain of the MHC Class 1 is important for positive selection.

Class 2 negative transgenic mice were created by either deletion of the Class 2 promoter region [113, 114] or through mutation in the invariant chain [115]. Spleen, peripheral lymph node and thymic cells of these mice were shown to be Class 2-/- although their physiology was unaffected. Class 2-/- mice had fewer CD4+8- thymic cells compared to Class 2+ve mice although some CD4+8- cells were present. These cells expressed markers of immaturity eg. J11D and had a cortical location. Some also expressed low levels of TCR and CD8. In the periphery of Class 2-/- mice CD4+8- cells were present but in reduced numbers. CD4 and TCR expression was also reduced [113] and these cells failed to reject allografts [114]. In all the Class 2-/- mice there was increased numbers of single positive CD8 cells. Thus the absence of Class 2 inhibited positive selection of CD4+ cells.

Class 1 negative mice were created by disrupting the $\beta 2$ microglobulin ($\beta 2m$) gene [110, 111] or through the disruption of the TAP-1 gene (transporter associated with antigen processing) [112]. Homozygous and heterozygous $\beta 2m$+ve mice had equivalent numbers of DN, DP and CD4+8- cells however in homozygous $\beta 2m$-/- mice the CD4-8+ cell population was significantly reduced. Thus the absence of Class1 inhibits positive selection of CD4-8+ cells.

Similar observations were made in TAP-1-/- mice [117].

From these data it was concluded that although MHC expression was not involved in expression of the co-receptor (CD4+8+ cells were present in Class 1 and 2 -/- mice) it was important for positive selection and differentiation into single positive mature cells.

iv) The importance of peptide bound to MHC in positive selection.

From the experiments described above positive selection of antigen-specific tg TCR T cells occurred in the absence of the protein for which it was specific. However it has been shown that each MHC molecule presents a distinct allele specific peptide motif [118]. It has also been shown that from the HLA-A2.1 MHC molecule 200 different species of peptides were bound [119].

The relevance of the peptide has been shown in transgenic mice which express variants of the MHC molecule shown to induce positive selection for tg TCR cells [120-123]. Mutations have been induced in the α1 and α2 regions (H-2K^{bm1}, H-2K^{bm3}) which interact with the TCR and in the β sheet region (H-2K^{bm8} and H-2K^{bm5}) on which the peptide is presented. Nikolic-Zugic and Bevan (1991) [120] investigated the selection of H-2K^{b} specific TCR+ve cells capable of recognising ovalbumin (OVA) in the context of H-2K^{b} in mice with the forementioned MHC mutations. No positive selection occurred in the MHC H-2K^{b} mutant mouse in which there has been substitution of amino acids on the β sheet facing into the MHC molecule.

Crossing mice possessing a transgenic TCR specific for LCMV in association with H-2D^{b} with H-2D^{b} mutant mice (H-2^{bm13} and H-2^{bm14}) resulted in no positive selection when the H-2^{b} MHC molecule had mutations in the α helix (H-2^{bm14}) which interacted with the TCR. However positive selection was enhanced in mice possessing mutations in the β sheet (H-2^{bm13}) [121]. These observations were in contrast with the results of Jacobs et al (1990) [123] who crossed H-Y specific TCR tg mice with simliar H-2 mutant mice. Positive selection did not occur if there was a mutation in the peptide or TCR specific regions of the MHC. Positive selection also did not occur for the 2C tg TCR cells in the presence of an H-2K^{b} molecule which had mutations in part of the molecule expected to be involved in peptide binding [122].

Thus mutations in the peptide binding site inhibit positive selection of MHC specific TCR+ve cells indicating the importance of peptide in positive selection.

The role of peptide in postive selection was also shown in Tap-1 -/- fetal thymic organ cultures (FTOC) [117]. As described earlier Tap-1-/- mice are defective in the positive selection process, probably due to the lack of MHC Class 1 on the cell surface of these mice.

Fetal thymic lobes from day 16 Tap-/- transgenic mice were given various peptides, known to induce the surface expression of various Class 1 alleles, on the surface of Tap-/- spleen cells, in the presence of exogenous β_2m. 10 days later the lobes were disrupted and the levels of Class 1 and 2 were measured. It was observed that peptides induced specific MHC surface expression on FTOC cells; OVA peptide $OVA_{257-264}$, influenza peptide $IF_{366-374}$ and sendai virus nucleoprotein (NP) peptide SV $_{324-332}$ induced surface expression of D^b and K^b whilst vesicular stomatitis virus NP peptide VSV_{52-59} induced K^b expression.

In TAP-/- FTOC the percentage of CD8+ve cells was reduced as compared to TAP+/+ cultures due to a defect in positive selection. After addition of IF and OVA peptides the level of CD8 in FTOC Tap-/- lobes increased above background whereas the SV and VSV peptides had no effect or variable effects, respectively, on CD8 expression. In this system it was found that each peptide-MHC combination selected a discrete CD8+ve T cell population.

Over 1000 naturally occurring peptides have been extracted from C57BL/6 thymic cells. Adding this mixture of self peptides to the Tap-1-/- FTOC also induced the appearance of a large number of polyclonal (as shown via the Vβ expression profiles) CD8+ve cells. Vukamovic et al (1993) [124] also noted this phenonomen using OVA recombinant peptide mixtures. The greater the diversity/complexity of peptides the more efficient CD8+ve selection.

These data suggest that the sole role of peptide is not just the stabilisation of MHC but that it is also important in selection.

v) The importance of CD4 and CD8 co-receptors in positive selection.

Early studies using monoclonal antibodies indicated the importance of CD4 and CD8 in selection. Anti-CD4 and anti-CD8 inhibits maturation of CD4+ and CD8+ cells respectively. The importance of these co-receptors has also been shown in transgenic mice.

Rahemtulla et al (1991) [125] created transgenic mice lacking CD4, through disruption of the CD4 gene by homologous recombination. These mice were healthy but the number of CD4 single positive cells was reduced and CD4 cell mediated responses and T dependent antibody responses were severally affected. Thus disrupting this gene inhibited positive selection of CD4+ve cells. In contrast the CD8 positive population in these mice were unaffected and were present in the periphery. Thus the expression of CD4 on CD4+8+ cells is not required for the positive selection of single positive CD8+ve cells. Mice over expressing the CD4 molecule (CD4++) have been created and have demonstrated the importance of CD4 in positive selection. Crossing these mice with H-Y TCR tg mice (specific for Class 1) resulted in CD4++/TCR tg Dbl-tg mice. The number of TCR transgenic CD8+ve cells in these mice was reduced as compared to TCR tg mice only and these CD8+ve cells were Class 2 restricted. They did not recognise the Class 1 D^b MHC molecule.

It was also found that the signaling transmembrane (TM) and cytoplasmic (CYT) region of CD4 was important in the commitment to a CD4+ve lineage [126]. This was shown in mice expressing the CD8+ve extracellular domain linked to the CD4 TM and CYT regions. Bone marrow from a cross between these mice and H-Y TCR tg mice was transferred into irradiated H-2^b recipient mice. These mice were found to possess Class 1 restricted CD4+ve cells.

The importance of CD8 in positive selection was also studied in transgenic mice. The CD8 gene was disrupted by homologous recombination in embryonic stem (ES) cells resulting in CD8-

/- mice. Homozygous CD8-/- mice lack single positive CD8+ve Class 1 restricted cells and they lack CTL responses. The CD4 developmental pathway in these mice seems unaffected by the absence of CD8 and they respond to MHC Class 2 alloantigens. There are normal numbers of DN and single positive CD4+ cells but there are no DP cells in the thymus thus the expression of CD8 is not required for CD4 development. Fung-Leung et al (1988) [127, 128] also showed that the CD8 molecule was important for positive selection. In the absence of the CD8 molecule, T cells possessing transgenic TCRs (H-Y or LCMV or 2C) specific for MHC Class 1 are not skewed towards CD8 positivity even in mice expressing the 'selecting' MHC Class 1 background. The level of CD8 expression also influences positive selection. Comparison of wild type (CD8+/+) and heterozygous (CD8+/-) mice showed a reduction in the number of CD8+ve cells expressing TCR.

Mice in which both the CD4 and CD8 were disrupted have also been created [129]. These mice have a developmental block at the DN stage although they have significant peripheral T cells which are TCR high and HSA-ve. This maybe due to the lack of positive selection since no CD4 nor CD8 molecules were present. Some positive selection must occur in the absence of these co-receptor since some TCR+ve cells reach the periphery. These cell recognise alloantigens in vivo and in vitro however self-MHC restricted recognition of either virus or minor histocompatibility antigens did not occur.

vi) Models for commitment to single positive CD4 and CD8 cells.

Two models seek to explain the commitment to and the emergence of mature single positive cells from the thymus: the instructive and selective models. Both models involve the interaction of TCR and co-receptor CD4 or CD8 molecules with MHC.

The instructive model suggests that positive selection and lineage commitment occurs at the DP stage of thymocyte development. Interaction of a Class 2 specific TCR with a

Class 2 molecule on thymic epithelial cells induces the down regulation of CD8 and the cell becomes a CD4 single positive cell. On the other hand interaction of a Class 1 specific TCR with a Class 1 molecule results in down regulation of CD4 and the cell matures into a CD8 single positive cell. This model predicts that CD4 +ve cells will be restricted to Class 2 and not Class 1 whilst CD8+ve cells restricted to Class 1 and not Class 2.

Various pieces of evidence have been put forward to confirm this model [130-132]. Borgula et al (1991) [130] created transgenic mice overexpressing the CD8α chain. Crossing these mice with H-Y TCR tg mice (Class 1 restricted) resulted in CD4 +ve cells, in female mice, which expressed CD8α tg capable of inducing signals. In these double transgenic mice, on a selecting background, only a small proportion of CD4+ve cells expressed the Class 1 restricted receptor.

Robey et al (1991) [131] also created mice in which the CD8 transgene is present on all T cells. Mature CD4+ve cells expressed the CD8 tg at levels equivalent to normal cells, whilst mature CD8+ve cells expressed double the amount of CD8. These CD8 tg mice were crossed with H-Y TCR tg mice, as above, and the presence of CD4+ve Class 1 restricted cells analysed. In female mice CD8 /TCR tg mice the presence of the CD8 transgene did not permit positive selection of CD4+ve Class 1 restricted cells. The CD4 positive cells present in these mice expressed Vβ regions known to preferentially interact with MHC Class 2. In these mice CD8 Class 1 specific TCR positive selection was not affected.

Swat et al (1992) [132] presented data which also suggested an instructive model. In the presence of a positive and negative selecting MHC background H-Y TCR tg cells (Class 1 restricted) and influenza (IF) TCR tg (Class 2 restricted) large DP TCR^{lo} cells were present. However small DP TCR^{hi} cells were only seen in mice with a positively selecting background. These small DP cells maybe a consequence of positive selection. The small DP cells in H-Y TCR tg mice, on a selecting MHC environment, had lower levels of CD4 whilst the

small DP cells in the IF TCR tg mice, on a selecting MHC environment, had reduced levels of CD8. This was also seen in vitro in the absence of thymic epithelial cells. Thus the ability of small DP TCR^{hi} cells to mature in the absence of TCR crosslinking indicated that these cells had already received a positive selection initiation signal. It was also noted that no CD4+ve cells with Class 1 specificity were obtained in vitro.

The second model to explain the lineage commitment is the selection model in which positive selection occurs not at the DP stage but at a later SP stage. In this model it is proposed that two steps involving TCR and MHC interaction occur. The first step initiates positive selection. This results in DP cells down-regulating either CD4 or CD8 randomly, stopping expression of RAG and Tdt, and upregulating the expression of mature T cell ligands e.g. CD69 [133]. From this model it was predicted that CD4+ve cells could express Class 1 restricted TCR and CD8+ve cells could express Class 2 restricted TCR. However if these cells do not receive the second signal from MHC Class 1 and Class 2 respectively, since they express the inappropriate co-receptor, they die. The second signal completes positive selection and results in CD4+ve and CD8+ve single positive cells with Class 2 and Class 1 specificity respectively. This model predicits that CD4+ve and CD8+ve cells which are Class 1 and Class 2 will be present as well as CD4 Class 2 and CD8+ve Class 1 immature intermediate cells.

In Class 2-/- mice [113] a population of unusual CD4+ve cells were found, about 30% of the normal number of CD4+ve cells in normal mice. These CD4+ve cells appeared to be at an intermediate stage between immature (they express low levels of CD8, HSA and have a cortical location) and mature (TCR^{hi} and express CD69) cells. This is the phenotype of positively selected cells suggesting that these CD4 cell express TCR specific for Class 1. In CD8-/- mice there are an equivalent CD8 intermediate population and this suggests that these cells maybe Class 2 restricted. This was investigated by Chan et al (1993) [134]. After crossing Class 1-/- and Class 2-/- mice only a few single positive CD4+ve and CD8+ve cells

were found in these double transgenic mice which may have been positively selected on non-classical MHC molecules. Most of the TCR^{hi} expression was on the DP cells. This result suggested that the CD4+ve cells present in Class 2-/- mice were restricted to Class 1 and that the CD8+ve found in Class1-/- mice were restricted to Class 2, since they are absent in double negative mice. These authors also investigated the existence of CD4+ve Class 1 specific cells in double transgenics lacking Class 2 but with TCR specificity for the H-Y antigen. CD4+ve T cells possessing a TCR specific for the H-Y antigen were found on a selecting but not on a non-selecting MHC background.

Davis et al (1993) [135] also presented data which fitted the selective model of positive selection. The selective model predicts that in mice which constitutively expressed a CD4 transgene Class 2 restricted CD8 cells would be rescued from cell death. They created two lines of transgenic mice expressing CD4 under the control of lck or CD38 promoters. The lck/CD4 and CD38/CD4 transgenic mice expressed high levels or normal levels of CD4 on immature thymocytes respectively, whilst in both cases peripheral mature thymocytes expressed CD4 at normal levels. These mice were crossed with β_2m-/- mice (Class 1 deficient) and the expression of CD8 was analysed. CD8(CD4tg)+ve cells were present in the thymus and lymph nodes of β_2m-/-/CD4+/+ double transgenic mice, regardless of the promoter (although sufficient rescue of these cells occurred best if the CD4 transgene was over expressed). These "rescued" cells had CTL activity and responded to allogeneic targets. They also expressed Vβ regions which were preferentially expressed on CD4+ve cells specific for Class 2, thus suggesting that these CD8+ve cells are Class 2 restricted.

The presence of CD8+ve Class 2 restricted cells was also shown in (CD4 tg x pigeon cytochrome c TCR tg +ve) double transgenic mice. Mature CD8+(CD4 tg +ve) cells in the thymus and periphery expressed pigeon cytochrome c tg TCR.

viii) Positive selection summary

In summary positive selection of thymoctes in the thymus results in mature CD8+ve T cells restricted to self MHC Class 1 and mature CD4+ve T cells restricted to self MHC Class 2. The process of positive selection involves the interaction of the TCR, MHC plus bound peptide and both CD4 and CD8.

1.5) Negative selection

The removal of self-reactive cells is thought to occur in the thymus. Developing thymocytes expressing TCRs with high affinity for the MHC/peptide complex are removed from the thymocyte pool by a process called negative selection. The major method of negative selection is deletion, 95% of developing thymocytes die in the thymus through a mechanism known as apoptosis. Self-reactive cells are also rendered unresponsive, anergic or they down regulate their TCR or CD8 co-receptor. The tolerance mechanism employed appears to depend on the antigen presenting cell that the self antigen is presented on: deletion if self peptide is presented by haematopoietic cells, whilst anergy and down regulation occur if antigen is on thymic epithelial cells.

Thymic tolerance is however an incomplete process since self reactive TCR positive cells can be found in the periphery of TCR transgenic mice usually having a DN or CD4-8^{lo} phenotype [136]. Tolerance to self proteins requires recognition of immunodominant epitopes plus MHC. It is possible that these "self-reactive" peripheral cell are T cells which escape thymic tolerance since they recognise subdominant or "cryptic" determinants plus MHC [137]. They could also be low affinity cells that are not deleted.

It has also been shown that although the cells susceptible to negative selection are the same as those undergoing positive selection, negative selection can occur in the absence of positive selection [138, 139].

In the following sections thymic tolerance is discussed.

i) Evidence for thymic negative selection

Early tolerance work focused on T cells, of 'normal' mice, utilising specific Vβ TCR elements which interact with Mls superantigens. These superantigens are encoded by the open reading frame (ORF) in the 3' long terminal repeat of endogenous and exogenous mammary tumour viruses [140]. Only certain Vβ TCRs can recognise Mls antigens presented by certain MHC Class 2 molecules, for example Vβ8.1 expressing T cells recognise Mls^a in association with $H\text{-}2^d$, $H\text{-}2^b$ and $H\text{-}2^k$ [141]. It was observed that T cells with Mls reactive TCR were eliminated in mice expressing Mls antigen, Mls^a mouse strains lack Vβ8.1+ve and Vβ6+ve [140-143] whilst Mls^c positive mouse strains lacked Vβ3+ve cells [140, 144, 145] and Etc-1 MTV-9 superantigen expressing mice lack Vβ5.2 and Vβ11 TCR positive T cells [146].

Thymic involvement in the removal of these T cells was shown in nude Mls^c mice: no Vβ3 deletion occurred [145]. Injecting neonatal mice with CD8+ve Mls^a+ve cells resulted in deletion of Vβ8.1 and Vβ6+ve T cells due to seeding of donor cells in the thymus [143]. It was also found that immature thymocytes expressed these Vβ regions whilst mature cells did not, thus suggesting that the thymus was involved in deletion [141, 142, 147].

Transgenic mice expressing rearranged TCR genes specific for an antigen provided further evidence that deletion of self-reactive cells occurred in the thymus [94, 148-153]. CD8+ve T cells expressing a transgenic TCR specific for the male antigen, H-Y, in association with $H\text{-}2D^b$ MHC were deleted in male transgenic TCR+ve mice expressing both this antigen and MHC whilst no deletion occurred in female transgenic mice lacking this antigen [148, 150]. Several authors [161][151, 154] have shown deletion of T cells bearing a transgenic TCR expressing Vβ8.1 in Mls^a expressing mice.

Van Ewijik et al (1988) [153] created transgenic mice expressing I-E genes in the cortex only or in the medulla and on peripheral macrophages. Analysing the frequency of Vβ17a expressing T cells in these transgenics revealed that deletion

of these cells occurred if I-E was on either cortical or medullary cells or both.

ii) At what stage does negative selection occur?

As described above deletion of Mls antigen specific T cells occurred in the thymus of normal mice. For most of these studies the deleted Vβ regions were found on the immature double positive cells whilst no expression was evident on mature single positive cells [141, 142, 147, 155]. These authors speculated that deletion occurred at a late stage of double positive development or in the transition from DP to SP cells. This was also confirmed by transgenic mice. Pircher et al (1989) [154] and Blackmann et al (1990) [156] showed that in Mls^a expressing mice double positive T cells expressing transgenic Vβ8.1+ve TCRs were present however Vβ8.1 TCR +ve SP T cells were reduced. Mice expressing a transgenic TCR specific for cytochrome c in association with Class 2 I-E or I-A have variable levels of DP cells but lacked CD4+ve single positive cells [157].

The stage at which negative selection occurs has also been analysed using in vitro assays [158, 159]. Day 14 thymic lobes from a 2C TCR transgenic mice specific for pigeon/moth cytochrome c bound to MHC Class 2 $I\text{-}E^k$ were dissociated and cultured with moth cytochrome c. Here elimination was shown to occur before or immediatley after CD4 and CD8 expression, thus this peptide induced tolerance in the DP thymocytes. A peptide not recognised by the 2C T cells did not induce deletion. By varying the concentration of peptide added to the cultures it was found that double positive immature T cells are deleted at very low peptide concentrations. The work of Vasquez et al (1992) [157] also indicated that deletion of DP cells was dose dependent: immature thymocytes are more sensitive to antigen than mature T cells despite having fewer ligands. Significant deletion of 2C TCR expressing DP cells occurred when moth cytochrome c concentration was at 0.025μM. This concentration of peptide did not activate mature 2C TCR+ve T cells.

Male H-Y TCR transgenic mice [150] have a dramatic reduction in the double positive thymic population suggesting that it is at this stage of thymocyte development that deletion occurs. Takahama et al (1992) [160] suggested that the reduction in DP cells in the H-Y TCR transgenics was due to the inhibition of CD4-8^{lo} precursor development and that these cells are the targets for negative selection. 50% of CD4-8^{lo} H-Y TCR+ve thymocytes from transgenic mice developed into CD4+8+ cells in culture and this was blocked by anti-TCR antibodies and by male APC from male H-2^{b} nude mice but not female mice. Similarly in vivo, precursor fetal thymocytes from male, expressing H-Y and appropriate MHC, but not female transgenic mice did not develop into DP cells in vitro. It was also noticed that these CD8^{lo} immature precursors (HSA^{hi} and ICAM^{hi}) expressed CD5, which was associated with antigenic stimulation, thus it appeared that antigenic stimulation inhibited the development of the DP cells. Since CD8^{lo} immature cells did not accumulate, nor did they undergo apoptosis, it was hypothesised that these cells became the DN cells which are found in large numbers in these mice. Swat et al (1991 and 1994) [158, 161] have published conflicting data. In vitro culture of CD4+8+ thymocytes from female H-Y transgenic in the presence of male APC from thymus or spleen resulted in deletion of these DP cells such that after 48 hours in culture few DP cells remained. This was not seen if APCs expressed the wrong MHC or lacked the male antigen [158]. CD4-8^{lo} immature thymocytes from a non-selecting H-Y TCR transgenic male mice in the absence of an antigenic signal developed into CD4+8+ cells. In contrast, in the presence of male cells (antigenic stimulus) there is a reduction of DP cells and no increase in DN cells was seen. However this stimulus did not entirely prevent DP generation. It was concluded that deletion either occurred before or after the expression of CD4 and that deletion occurred at the DP stage and not at the CD4-8^{lo} stage as described above.

Overall, these data suggest that deletion of T cells occurs at the DP stage, early or late, or as they become single positive

cells. The difference in timing appears to depend on the TCR expressed and the antigen involved. This was confirmed further by Picher et al (1989) [152] using double antigen specific TCR transgenic mice. The TCR of these mice recognises LCMV and Mls^a. In Mls^a TCR transgenic mice no reduction in DP cells occurred, as found in earlier studies, however there was a reduction in the SP TCR+ve cells. In contrast LCMV infected TCR transgenic mice had deleted DP cells. This difference may be due to the time dependent appearance of the antigen and localisation of antigen, as also seen by Zal et al (1994) [162]. Transgenic mice expressing TCR receptors for self circulating C5 antigen (C5 is the fifth component of mouse complement: a serum self protein) [163] developed tolerance late in ontogeny and DP cells were unaffected. This was attributed to the fact that dendritic cells in the CM junction presented C5 which would involve uptake, processing and presentation. In the transgenic mice expressing TCR's specific for both LCMV and Mls^a, interaction with LCMV in the cortex and medulla and with Mls^a in the outer cortex medulla may affect the cells that undergo deletion. Another possiblity is that high affinity interactions delete LCMV specifc cells whilst low affinity interactions delete Mls^a specific cells.

iii) AFFINITY, AVIDITY AND PEPTIDE

The data above suggests that cells undergoing negative selection are similar to those undergoing positive selection. One hypothesis to explain this is that selection can be attributed to affinity; thymocytes with low affinity for MHC are positively selected whilst those of high affinity are deleted. An alternative hypothesis is the peptide model where both positive and negative selection requires high affinity TCR-MHC interaction. However the peptide/ MHC complex that mediates selection differs; bone marrow derived cells present a ubiquitous peptide whilst thymic epithelia present both ubiquitous and unique peptides.

There have been several models utilising transgenic mice which confirm the affinity model [164, 165]. On a selecting MHC background, H-2K^b, 2C TCR transgenic positive T cells were positively selected. When these transgenic mice were mated with CD8 transgenic mice (where CD8 is under the control of CD2 promoter element) the fate of the 2C TCR receptor positive T cells changed from being positively to negatively selected and in the thymus the total number of thymocytes were reduced. These authors hypothesised that if increasing the levels of CD8 induced negative selection then the remaining cells would have low CD8 levels. This was the case. This study was in contrast to H-Y transgenics where increasing CD8 increased the efficiency of positive selection. The authors suggested that H-Y specific TCRs were of very low affinity and that increasing CD8 levels increased affinity but not to the level required for negative selection.

2C TCR transgenic mice were also crossed with transgenic mice expressing distinct and elevated levels of CD8, either 2, 3 or 6-10 times more than normal [166]. T cells in mice expressing the 2C TCRs plus high or intermediate levels of CD8 were deleted whilst those with low CD8 levels were selected to mature. Decreasing the affinity of the CD8 and MHC Class 1 interaction allowed T cells to escape deletion [167].

In another experiment the CD2 promoter was used to direct Vβ11 expression. Two lines of mice were created; one with 'low' Vβ11 expression, another with 'high' Vβ11 expression [165]. CD4Vβ11+ cells are usually deleted in the presence of MMTV 8, 9 and 11 however no deletion of CD4Vβ11+ve cells occurred in the 'low' Vβ11 expressing mice and these cells were found in the periphery. In contrast CD4Vβ11+ve cells were deleted in the 'high' Vβ11 expressing mice. These data suggest that high avidity cells are deleted whilst those of low avidity escape and are found in the periphery. Similar observations were made in TCR transgenic mice specific for murine haemoglobulin/I-E^k and expressing this ligand [168].

<u>In vitro</u> FTOC using transgenic TCR thymocytes specific for LCMV also supported the affinity and avidity model. Low

concentrations of peptide induced positive selection whilst high concentration resulted in tolerance [169, 170].
The peptide recognised by self-reactive cells is also important. Cabaniols et al (1994) [137] suggested that high affinity clones recognising the immunodominant peptides of HEL are negatively selected even in the presence of a low dose of antigen whilst those which have low affinity specific for cryptic peptides are not.

iv) Contribution of CD8 and CD4

As described above tolerance involves the removal of T cells with TCRs of high affinity. What is the role of CD4 and CD8 co-receptors in tolerance induction?
The role of CD8 was analysed by crossing H-Y, LCMV and 2C TCR transgenic mice with CD8-/- mice. It was found that there was a differential requirement for CD8 in deletion: no deletion of H-Y and LCMV TCR transgenic cells occurred in the absence of CD8 whilst deletion of the 2C TCRs occurred even in the absence of this co-receptor [128]. This result was explained with respect to affinity: cells with high affinity TCRs, for example the 2C TCR, do not require CD8 interaction with Class 1 for deletion, whilst cells with low affinity TCRs, for example H-Y and LCMV TCRs, require CD8 interaction with MHC Class 1 for deletion to occur. Further evidence for a lack of CD8 involvement in deletion was shown by Knobloch et al (1992) [171]. Crossing Class 1 mutant mice with TCR transgenic mice specific for K^b resulted in deletion of DP cells suggesting that the missing interaction between Class 1 and CD8 did not prevent negative selection.
CD8 was also shown to be required for negative selection in mice expressing MHC Class 1 with $\alpha 3$ domains which do not interact with CD8 [172].
Deletion of Vβ6 or Vβ8.1 TCR expressing T cells in Mls^a mice was prevented when mice were treated with anti-CD4 monoclonal antibodies [173], thus CD4 appears to be important in tolerance induction. However like CD8, CD4 was shown to be a differential requirement for deletion [174]. Vβ TCR

expression was analysed in CD4-/- knockout mice backcrossed onto an Mls^a environment. Vβ6, 9, 8.1 CD8+ve T cells were deleted whilst Vβ7 expressing cells were not. This data suggested that T cells expressing TCRs with high affinity for Mls^a are deleted without the participation of CD4 whilst T cells expressing TCRs of low affinity, as is the case with Vβ7, require CD4 interaction with Class 2 for clonal deletion [174]. CD4 and CD8 positive cells are also associated with $p56^{lck}$. However inhibition of this molecule did not interfere with deletion [175].

v) Bone marrow cell types involved in thymic tolerance.

Bone marrow chimaeric experiments have suggested that bone marrow derived cells other than T cells are involved in thymic tolerance [176, 177] although thymocytes themselves have also been shown to induce thymic tolerance [178, 179]. FACS purifed CD4+8+ immature thymocytes from $H\text{-}2D^b$ mice expressing transgenic TCR specific for LCMV plus $H\text{-}2D^b$ under went apoptosis when cultured with LCMV. Thus antigen presenting immature DP thymocytes can tolerise antigen-reactive immature thymocytes in vitro by deletion induced apoptosis 6-8 hours after antigen exposure [178]. Thymocytes were also shown to induce tolerance in vivo. Simpson et al (1993) [179] created transgenic mice expressing $H\text{-}2K^b$ under the control of the human CD2 gene such that all thymocytes and peripheral T cells expressed this antigen. These mice were tolerant to $H\text{-}2K^b$ skin grafts.

Pircher et al (1993) [138] suggested that negative selection did not require a specific APC. This was also shown by Iwabuchi et al (1992) [180] using an in vitro single cells assay in which thymocytes expressing LCMV specific TCRs were deleted after being co-cultured with various APCs and LCMV peptide. These experiments indicated that a whole host of cells were capable of inducing deletion, including embryonic and transformed fibroblasts, melanoma cells, cortical thymic epithelial cells, neural cells, TNC as well as macrophages, and DC from the thymus and spleen. Tolerance was induced in a

dose dependent manner with some cell types requiring nanomolar concentrations of antigen whilst splenic macrophage and DCs required only picomolar amounts. It was also found that there was a minimum amount of antigen required for negative selection. These data led the authors to suggest that the stage of thymocyte development, rather than the type of APC cell or antigen concentration, was important for deletion [180].

Many studies have highlighted the efficiency of DC in tolerance induction; a) thymic dendritic cells were x10 more efficient than thymic macrophages at inducing in vivo and in vitro tolerance [138], b) deletion of Vβ6+ve T cells occurred in Mls^b FTOC reconstituted with Mls^a DC and B cells and Mls^b thymocytes (here the presence of B cells was important since it is thought that these cells pass the Mls^a antigen to Class 2 positive cells) [181], c) male DC induced deletion of DP H-Y specific thymocytes in vitro [182] and d) DC from C5 expressing mice stimulated C5 specific transgenic TCR+ve thymocytes since they retain C5 on their surface for a long period of time [163]. The properties attributing to the tolerising ability of DC are: a) their high Class 2 expression b) slow turnover of membrane Class 2 and c) adequate antigen internalisation [163].

The role of macrophages in tolerance induction was investigated in mice expressing an I-E transgene on macrophages [183]. Expression of I-E reactive Vβ5, 11 and 17a T cells were examined in these mice. No deletion occurred. The remaining cells were anergic. This confirmed the finding of Pircher et al (1993) [138] that splenic macrophages were more effective than their thymic equivalents at tolerance induction. B cell effectivness in tolerance induction was shown by Aiba et al (1994) [184]. Murine thymic Vβ8+ve lymphocytes in the presence of SEB and a B cell lymphoma expressing Class 2 at high levels undergo deletion. However when these cells are fixed clonal deletion was inhibited.

Mature thymocytes have been shown to require a second signal to undergo proliferation: crosslinking the TCR does not induce

proliferation. However signalling through CD28, via interaction with B7 on APCs, does induce proliferation. A second signal for thymocyte deletion has also been observed [184, 185]. Crosslinking the transgenic TCR from DP T cells specific for moth cytochrome c with murine Class 2 transfected fibroblasts plus antigen results in deletion via apoptosis. When the TCR was crosslinked in the absence of APC no deletion occurred but the CD4+8+^{hi} cells down regulated their CD4 and CD8 co-receptors and became CD4+8+^{lo}. On addition of APCs deletion occurred [185]. This second signal was not provided by the B7/CD28 pathway, since blocking these receptors did not stop deletion [185-187] and CD28 deficient mice still undergo deletion. Other signals that may be involved include LFA-1/ICAM1, CD2/LFA3 and VLA4/VCAM. Antibodies to LFA-1 and ICAM1 have been shown to interfere with tolerance induction [186] but the murine fibroblasts used by Page et al (1993) [185] did not express LFA1 or ICAM1 and tolerance still occurred. CD2 has also been ruled out since disruption the CD2 gene did not interfere with thymic development [188].

vi) Thymic epithelium and tolerance

Thymic epithelial components have also been shown to be involved in negative selection either through deletion of self reactive cells or by inducing unresponsiveness.

Bone marrow chimaeras were constructed such that the antigen recognised by a specific Vβ bearing T cells was presented on either the thymic epithelia or on donor bone marrow derived cells [177]. When H-2^{k} expression was limited to bone marrow derived cells Mls^{a} deletion of Vβ17a and Vβ6 was found. However when H-2^{k} expression was limited to the recipient thymic epithelia then very little deletion of these Vβ+ve cells occurred. These remaining cells did not respond to Mls^{a} but did respond to a third party antigen and anti-TCR crosslinking. Similar results were found by Roberts et al (1990) [189]. I-E expression only on thymic epithelial cells did not result in deletion of Vβ17a+ or Vβ6+ cells and the

remaining cells did not respond to either Mls^a or I-E or anti-TCR crosslinking. Thymic epithelia therefore appears to induce anergy. A role for thymic epithelial cells in anergy induction was also seen using transgenic mice [176, 190]. H-$2K^b$ expression was directed to the mammary tissue (KAL) [190] or keratinocytes (Kb) [176]. These mice did not reject H-$2K^b$ positive skin despite its alloantigenic nature. Crossing these H-$2K^b$ mice with mice possessing anti-H-$2K^b$ TCRs indicated that this tolerance was not due to deletion of DP self-reactive cells nor was it due to down regulation of CD8 or TCR. However unlike the anti-H-$2K^b$ single transgenics no K^b-reactive CTLs, even in the presence of rIL-2, occurred in these double transgenic mice. These cells were also either non responsive [190] or responsive [176] to anti-TCR antibodies with or without rIL-2. Tolerance in KAL transgenic mice was attributed to expression of H-$2K^b$ by thymic epithelial cells as shown via bone marrow chimaeras and skin graft rejection experiments whereas tolerance in the Kb transgenic mice was attributed to both bone marrow and thymic epithelia H-$2K^b$ expression [176]. These experiments suggest that thymic epithelia are not capable of inducing deletion of self-reactive T cells.

In contrast several authors have produced data which support the role of these cells in clonal deletion [191-193]. Bone marrow chimaeras in which I-E was expressed on host epithelia cells resulted in deletion of Vβ6+ve cells. Negative selection induced by thymic epithelial cells occurred after or during the DP to SP transition. Tolerance induction by thymic epithelial cells is not as efficient as tolerance induction by bone marrow derived cells; for example, T cells expressing low levels of TCR Vβ6+ve regions are found if tolerance is induced by the former rather than the latter [193].

Bone marrow chimaera studies involving TCR transgenic agreed with these data [191]. Mice expressing I-E on the thymic medullary epithelia induced partial deletion of transgenic Vβ5+ve T cells. However no deletion of transgenic Vβ11+ve T cells occurred. These observations were explained

by the fact that Vβ11+ve T cells required the high density of I-E on bone marrow cells to induce tolerance [192]. Thymic epithelial cells expressed I-E at a low densities.
Antigen expression on thymic epithelia has also been shown to induce what is known as "split-tolerance" such that deletion is found in vivo but not in vitro [194, 195]. This may be due to deletion of high affinity clones whilst low affinity clones escape tolerance and are activated in vitro.

vii) TCR and CD8 down regulation

Tolerance can be associated with down regulation of the TCR rather than deletion [196]. KAL transgenic mice expressing H-2K^b in the mammary gland are tolerant to H-2K^b skin grafts. Tolerance was shown to be due to expression of H-2K^b in the thymus. Crossing these mice with mice possessing T cells with anti-H-2K^b transgenic TCRs indicated that tolerance was not due to deletion: anti-H-2K^b expressing T cells were present in the resulting double transgenic mice. Tolerance was associated with the down regulation of transgenic TCRs on T cells. Down regulation of CD8 has also been found in chimaeric mice which expressed male H-Y antigen on thymic epithelia and not on bone marrow derived cells [197]. In this situation female H-Y transgenic cells developing in chimaeric thymic environment are tolerant to the H-Y antigen not through a process of deletion of DP cells.

viii) Programmed Cell Death/Apoptosis

Cells which recognise self peptide plus self MHC with high affinity are removed from the thymocyte pool, as described. This process involves the programmed death of cells, or apoptosis, which is characterised by DNA fragmentation, chromatic condensing and T cell shrinkage. Double positive thymocytes from transgenic mice expressing a TCR specific for chicken OVA plus I-A^d undergo apoptosis if OVA is injected into these mice and this occurs within the thymus subcapsular and deep cortical areas [198]. Cells undergoing cell death show a marked change of surface phenotype [199]. CD4, CD8 and HSA

expression are decreased whilst TCR β/CD3, CD69 and CD25 expression increase. This may reflect a breakdown in intracellular trafficking leading to an accumulation of markers in the cell membrane [199].
The FAS cell surface protein has been shown to mediate apoptosis and is expressed throughout thymocyte development. Ogasawara et al (1995) [200] showed that both DP and SP thymocytes expressed the same amount of FAS however following an anti-FAS intraperitoneal injection DP thymocytes are deleted due to apoptosis. Despite the high expression of FAS in SP cells these cells are resistant to FAS mediated apoptosis. This may be due to the expression of bcl-2, a molecule which promotes survival of cells derived of essential growth factors in vitro. Its role in thymic tolerance has also been studied [201-204]. Crossing female H-Y transgenic mice on a non-selecting background with bcl-2 transgenics resulted in survival of DP cells in these mice. These cells did not differentiate further. Introducing the bcl-2 gene into male H-Y transgenics increased the number of DP cells 4-6 fold compared to mice lacking bcl-2 and over expression of bcl-2 reduced the efficiency of DP deletion. Self-tolerance was still maintained in these mice since peripheral T cells did not respond to the H-Y antigen [201]. Similar data were obtained by other authors [202-204].

ix) Negative selection summary

Removal of T cells expressing TCRs capable of recognising self peptide/MHC complex with a high affinity occurs within the thymus. This is known as negative selection. T cells undergoing negative selection die by apoptosis, or they are rendered anergic or they down regulate their TCR or CD8 co-receptor. Various factors have been shown to influence this process including antigen, CD4 and CD8 co-receptor, MHC and TCR specificity.

1.6) Peripheral tolerance

Workers investigating tolerance to alloantigens expressed in the periphery have looked mostly at the expression of MHC and viral proteins in tissues such as the pancreas and liver, as well as on erythroid cells. Targeting of genes to these organs involves the use of tissue specific promoters: expression of a transgene in the pancreatic islets involves the use of either the rat (RIP) or human (HIP) insulin promoter and enhancer elements, whilst expression on the acinar cells in the pancreas or liver is directed by either the elastase promoter and enhancer or the zinc inducible metallothionien promoter, respectively. Controlling elements of the human beta-globin gene have been used to direct expression of transgenes to RBCs. In the following sections tolerance in such transgenic mice is discussed with respect to the expressed transgene and it's tissue distribution.

i) MHC Class 1 transgenics and erthyroid expression [205]
H-2K^{k} mice expressing transgenic H-2K^{b} on 50% of their red blood cells accepted K^{b} skin grafts and no K^{b} CTLs were observed *in vitro* even in the presence of IL-2. These observations suggested that these mice were tolerant to H-2K^{b}. The mechanism of tolerance was not one of deletion since lymph node cells from these mice proliferated to H-2K^{b} expressing stimulators to the same extent as non-transgenic mice regardless of the presence of IL-2. Tolerance was not due to H-2K^{b} expression in the thymus since no expression of this protein was found in this organ. However positive selection had occurred resulting in H-2K^{b} restricted T cell responses.

ii) MHC Class 1 expression in the pancreatic beta cells or acinar cells.
The expression of Class 1 on pancreatic cells and its role in diabetes was analysed in H-2K^{b} Class 1 transgenic mice. Diabetes occurred in some mice expressing transgenic H-2K^{b} on pancreatic tissue [206-210]. Expression of transgenic Class 1 on these cells caused diabetes by interfering with insulin production rather than inducing destruction of pancreatic islet

cells by an immune response. No immune infiltrate was observed even after priming with H-2K^b spleen cells [210]. The lack of an immune response to the transgenic MHC suggested that these mice were tolerant to this alloantigen. Spleen cells from unprimed Class 1 transgenic mice did not kill H-2K^b targets in vitro without addition of exogenous IL-2 [206]. Thus transgene specific cytotoxic cells remained in these mice but in a tolerant state. Their existence was confirmed in double transgenic mice expressing H-2K^b on pancreas islet cells and a transgenic T cell receptor specific for H-2K^b [208, 210]. The H-2K^b TCR specific T cells in single transgenic mice rejected H-2K^b skin grafts rapidly compared to non-transgenic littermates and strong anti-H-2K^b CTL responses were generated in vitro [208]. Double transgenic mice had similar numbers of TCR transgenic positive T cells in both the thymus and periphery but unlike the single TCR transgenic mice this large potential pool of auto-reactive T cells did not reject H-2K^b skin grafts. Cytotoxic T cells from these mice were generated at a less efficient rate than in non-transgenic mice. The mechanism of tolerance appeared not to involve deletion of self-reactive clones nor was it due to TCR and CD8 down regulation, since expression levels of TCR and CD8 per cell were similar to non-transgenic animals [208]. The mechanism favoured was one of clonal silencing or anergy. As the pancreatic cells died tolerance subsided suggesting that the continuous presence of antigen was required for the maintenance of the tolerant state.

Deletion of T cells specific for antigens expressed in the pancreas has been documented [211]. H-2L^d transgenic mice expressing this protein on the surface of pancreatic acinar cells (without thymic or splenic expression) were tolerant to H-2L^d. Crossing these mice with mice expressing a transgenic receptor specific for H-2L^d, the 2C TCR transgenics, resulted in double transgenic mice which were also tolerant to this MHC molecule. Tolerance in these mice was found to be the result of elimination of 80% of the 2C TCR+ve T cells. Those cells escaping elimination had reduced H-2L^d activity and

were in an anergy state. Anergy was not associated with a period of proliferation nor was it associated with the down regulation of TCR or CD8.

Why should both Class 1 expressing pancreatic models have different outcomes? Both the transgenic MHC molecule and the transgene expressing cell are different in these models. It is possible that antigen presentation by pancreatic acinar cells results in deletion whilst antigen presentation by pancreatic islet cells results in anergy. Another explanation is that the affinity of the T cell dictates the outcome. For example, high affinity T cells in the H-2L^d model are deleted upon recognition of the transgene in the pancreas whilst T cells with a lower affinity for H-2L^d are rendered anergic. In contrast, high affinity T cells specific for H-2K^b may have been deleted in the thymus (due to a few molecules of H-2K^b being expressed in this tissue) [217] such that the remaining cells are only susceptible to silencing following contact with H-2K^b in the periphery.

iii) MHC Class 1 expression on neuroectodermal, hepatocyte and keratinocyte cells

Expression of a H-2K^b transgene under the control of either the glial fibrillary acidic protein promoter, albumin promoter or keratin IV promoter was directed to neuroectodermal (GFAP-K^b transgenics), hepatocytes (ALB-K^b transgenics) and keratinocytes (KIV-K^b transgenics) cells respectively [212-214]. Each of these mice displayed distinct peripheral tolerance mechanisms.

GFAP-K^b transgenic mice express H-2K^b in cells of neuroectodermal origin including Schwann cells in the intestine and epithelial cells of the choroid plexus as well as cells in the brain. ALB-K^b transgenic mice expressed H-2K^b in the liver (at different levels depending on the transgenic line) and KIV-K^b transgenic mice expressed H-2K^b on epithelial cells of the tongue, skin and footpad. No H-2K^b expression was found in the thymus of any of these transgenic mice. All 3 transgenic mice accepted K^b skin grafts. In addition GFAP-K^b

transgenic mice also accepted the EL-4 tumor cell line which expresses H-2K^b, and these mice did not show a primary anti-K^b CTL response in vitro. These mice were mated with mice expressing a transgenic TCR (recognised by a clonotypic antibody) specific for H-2K^b. Despite the presence of many self-reacting cells the resulting double transgenic mice were tolerant to H-2K^b. They all accepted H-2K^b skin grafts and GFAP-K^b double transgenic mice retained their ability to support the growth of the EL-4 cells. No differences in tolerance levels were found in the three ALB-K^b lines expressing the transgene at different levels, suggesting that the density of self antigen does not play a crucial role in tolerance.

In GFAP-K^b and ALB-K^b double transgenic mice the number of CD8+ve clonotypic+ve cells was reduced in both spleen and lymph nodes compared to control mice (the transgenic TCR-only mice). No reduction in the number of CD4+ve clonotypic +ve cells was found. Analysis of the TCR-ve CD2+ve cells, following CD4 and immunoglobulin positive cell removal, indicated a decrease (about 60%) in the number of cells expressing TCR in the double transgenics as compared to single transgenic TCR+ve mice. These data suggested that the mechanism of tolerance in these mice was one of TCR down regulation.

In vitro culture of T cells from GFAP-K^b double transgenic mice with H-2K^b spleen cells resulted in the re-expression of TCRs although this did not occur with ALB-K^b T cells even after IL-2 stimulation. Re-expression of TCRs needed CD2 crosslinking.

In contrast KIV-K^b double transgenic mice did not show any evidence of deletion, anergy or down regulation of TCR. H-2K^b reactive T cells in these mice had enhanced levels of activation markers, such as CD44 and CD2, suggesting that these cells had come in contact with antigen [214]. Tolerant cells in the KIV-K^b double transgenic mice were long-lived and tolerance persisted only with continuous contact with the tolerogen.

The differences in tolerogenic mechanisms seen between these mice was attributed to; 1) different tolerogenic signals provided by various tissues, 2) varying numbers of antigen-bearing cells within a certain tissue, 3) accessibility of the organ, 4) different adhesion molecules on cells and 5) the intensity, frequency and length of contact.

Down regulation of both TCR and CD8 was also seen in double transgenic mice expressing H-2K^b under the control of an LPS inducible human complement reactive promoter (CRP-K^b) and H-2K^b transgenic TCRs on T cells [215] These (CRP-K^b x anti-K^b TCR) double transgenic mice were tolerant to H-2K^b despite the extremely low levels of H-2K^b in the liver. A reduction in H-2K^b specific T cells was observed in these mice apparently due to down-regulation of CD8 and TCR. Further down regulation of these molecules occured following the increase in H-2K^b levels through LPS stimulation. 4 weeks following LPS stimulation H-2K^b levels decreased which resulted in the reappearance of H-2K^b specific T cells, confirming the reversibility of this tolerance mechanism. From this study it appeared that tolerant cells can be driven deeper into a state of tolerance depending on antigen dose.

This susceptibility to additional tolerising signals has been described as the multistep model of T cell tolerance [216, 217]. Schonrich et al (1994) [216] found that T cells expressing transgenic H-2K^b reactive TCRs were not deleted in double transgenics expressing a mutant form of H-2K^b (K^{bm1}) although they were partially tolerant: <u>in vivo</u> they did not reject H-2K^b skin grafts but <u>in vitro</u> they were reactive to K^b-positive splenocytes. <u>In vitro</u> activity was abolished following intravenous injection of K^b positive cells or when (anti-H-2K^b TCR X K^{bm1}) were crossed with ALB-K^b mice. Contact with K^b on hepatocytes led to deletion of the tolerant T cells.

Mice expressing a soluble form of Q10 (a non-classical Class1 encoded molecule) in the liver [218-220] with serum levels of 10-60μgs/ml were not tolerant to Q10 since H-2 unrestricted CTL activity to membrane bound Q10 was found [218]. However mice expressing membrane bound Q10 protein on the surface of

liver parenchymal were tolerant to this molecule [219, 220] with no evidence of cellular infiltrate into the liver before or after deliberate immunisation with this protein [219, 220] or cross-reactive antigens [220]. Following in vitro activation of CTLs however infiltration was observed [220]. Tolerance in this model was stable since transferring Q10 CTL cells into non-transgenic mice resulted in mice that were tolerant [219].

iv) Class 1 and H-Y TCR specific T cells.

The fate of CD8+ve Tcells expressing transgenic TCRs specific for the male H-Y antigen in female mice has been documented [139, 221-224]. Female mice positively select these cells such that CD8+ve T cells specific for the H-Y antigen are found in large numbers in the periphery of these mice. No negative selection occurs due to the lack of the H-Y antigen. Tolerance to H-Y was induced in the periphery following the injection of male lymphoid cells expressing H-Y antigens into adult [223] and neonatal female transgenic mice [139] or via transfer of transgenic female lymphoid cells into male nude mice [221, 222]. Exposure to male antigen resulted in a reduction in the number of H-Y reactive T cells through deletion [139, 221, 223]. CD8+ve H-Y reactive were shown to undergo apoptosis following intravenous injection into female mice [139]. Deletion was either preceded by a period of activation and proliferation, as shown through the expression of CD44, [221, 222] or it occurred rapidly without proliferation [139]. Deletion was however not complete since some CD8+ve male reactive cells remained in these models [139, 221-223]. These cells were found to be either; a) responsive to H-Y in vitro to 1/3 the level of non-transgenic mice [139, 223] or b) unreactive to H-Y or anti-TCR stimulation in vivo or in vitro [221, 222]. This anergy was however reversible as seen by "parking" experiments. "Parking" anergic CD8+ve male reactive T cells in female but not male mice for 3-8 weeks resulted in CD8+ve TCR transgenic cells capable of proliferating in response to rIL-2 and anti-TCR stimulation in vitro .Therefore in the absence of antigen stimulation, tolerance is reversible

although time is a critical factor. "Parking" cells for a few days did not reverse tolerance [222].
The importance of the CD8 molecule in peripheral tolerance was shown by Zang et al (1994) [223]. B6 H-Y transgenic female mice were injected with B6 male lymph node cells from CD4-/- (CD8+ve) or CD8-/- (CD4+ve) knockout mice. Only following the transfer of CD4-/- cells did reduction of male specific cells occur. As above CD8+ve H-Y reactive cells remained in the periphery of these mice and were fully responsive to male antigen in vivo [225].

v) Class 2 transgenic expression in the pancreatic beta and acinar cells

Class 2 molecules (both I-E and I-A) have been targeted to the pancreas using RIP and elastase promoters [226-234].
Although some I-E transgenic mice became diabetic this was not due to an immune infiltration of the pancreas [226, 227, 233], even after priming with the alloantigen. The suggestion that these mice were tolerant to the expressed I-E was confirmed by Markmann (1988) [229] by transplantation of transgenic I-E+ve pancreatic tissue to I-E-ve naive recipients: these mice accepted the I-E+ve graft. Gotz et al (1990) [226] showed that I-E reactive cells were present in I-E transgenic mice in vitro and in vivo despite the lack of autoimmune infiltration into the pancreas. These authors suggested that the I-E transgenic mice were not tolerant but that the immune system ignored the alloantigen due either to low I-E expression or lack of access to the pancreas. The hypothesis that I-E was hidden from the immune system was ruled out in another experiment: non-transgenic, non-tolerant, T cells destroyed the pancreas of I-E+ve transgenic mice [227].
The I-E molecule is recognised by T cells expressing either TCR Vβ17a and Vβ5 regions. In mice expressing I-E in the thymus T cells expressing these TCRs are deleted from the thymocyte pool. This however did not occur if transgenic I-E was expressed on either the pancreatic β [228] and on acinar cells [234]. The state of tolerance was thus not due to deletion

of self-reacting cells. The tolerance mechanism in these mice was not due to deletion of self-reactive T cells but due to anergy: cross-linking Vβ17a and Vβ5 positive cells from I-E tolerant mice resulted in a weak response as compared to non-transgenic controls [227]. T cells from these mice also responded weakly to irradiated I-E spleen targets [233].
Tolerance to I-A was also found in transgenic mice expressing I-A on the islet or acinar cells of the pancreas: no cellular infiltrate was found in these tissues nor did these mice reject I-A expressing alloantigenic skin grafts even after primimg with the alloantigen [225, 230-232]. Transgenic I-A was accessible to the immune cells since priming of transgenic and non-transgenic cells in vitro with I-A+ve cells resulted in pancreatic destruction in vivo and lymph node cells from these mice reacted strongly to I-A in-vitro [230-232]. Thus in this model it appeared that tolerant mice possessed T cells reactive to I-A in an inactive state [232]. Although deletion of self-reactive cells did not occur in these mice the authors did not rule out the possiblity that T cells with high affinity for I-A were deleted, whilst those with low affinity were not. The latter cells responding in the in vitro MLR assay [230].

vi) Viral transgenic mice with pancreatic expression.
SV40 [235-238], LCMV [239, 240], vesticular stomatitis virus glycoprotein (VSV-GP) [241] and influenza virus hemagglutinin (HA) [242, 243] antigens have been targeted to murine pancreatic β islets [236-239, 241-243] and acinar cells [235]. These transgenes had organ specific expression since no thymic expression was detected.
Transgenic mice (RIP-Tag) expressing the SV40 T/t antigen (Tag) in the pancreas were found to be either tolerant or non-tolerant to this transgene [235, 238]. No tolerance to Tag was found when RIP-Tag transgenic mice were crossed with TCR transgenic mice specific for this antigen in the context of MHC Class 1 H-2K^{k}. In these animals destruction of the pancreatic acinar cells occurred after immune infiltration [235]. This lack of tolerance was attributed to the late expression of the

transgene [235, 238] and was further investigated by Ye et al (1994) [237]. These author created two RIP-Tag transgenic mice: RIP-Tag2 and RIP-Tag4 [237]. The former mice being tolerant to Tag due to expression of the transgene during embryonic development. In contrast RIP-Tag 4 mice transgene expression occurred late in development at 10-12 weeks of age. These mice developed insulinomas and following immunisation produced, in vitro, anti-Tag CTLs. Once the transgene was expressed these mice became tolerant to Tag but anti-Tag CTLs were still produced in vitro [237]. Tolerance in these mice was not through deletion of self-reactive cells.
LCMV-GP transgenic mice expressing LCMV in the islet beta cells were crossed with LCMV-GP specific TCR+ve transgenic mice [239]. The resulting double transgenic mice were tolerant to LCMV and no diabetes occurred, even though positive selection of the LCMV CD8+ve cells was evident. 70-80% of the cells in these double transgenic mice expressed the transgenic TCR, and no TCR down regulation was evident. In vitro these cells proliferated in response to LCMV infected macrophages and could be stimulated by in vivo immunisation with LCMV-GP resulting in the development of diabetes. Thus in these mice a reversible state of anergy was evident.
Anergy was also shown to occur following injection of soluble LCMV-GP into LCMV TCR specific transgenic mice following a period of cellular proliferation [240].
Tolerance to HA and VSV-GP molecules in the pancreas has also been demonstrated [241, 242]. T_H cell tolerance to VSV-GP was shown by the inability to switch from IgM to IgG in VSV-GP transgenic mice [241] whilst tolerance in HA transgenic mice was shown by the lack of diabetes [242]. The latter mice were also tolerant to HA following in vivo priming with Vaccinia-HA and this was also shown in vitro following HA stimulation [242]. In contrast some HA transgenic mice developed diabetes. The lack of tolerance was attributed to low levels of HA on islet cells [243].

vii) Cytokines and peripheral tolerance.

Transgenic mice expressing the cytokines IFNγ [244] and IL-2 [245, 246] on pancreatic islet cells have been created. IFNγ transgenic mice become diabetic, due to a progressive destruction of pancreatic cells by the influx of inflammatory cells and lymphocytes from these transgenic mice are cytotoxic to normal islets. Crossing these tg mice onto a SCID mouse background resulted in no infiltration of the pancreas and no destruction of this tissue. The authors suggested that pancreatic expression of IFNγ results in the loss of tolerance to normal tissue by inducing the co-stimulatory activity essential for lymphocyte activation during an immune response [244]. Pancreatic infiltration by Ig+, CD4+ and CD8+ cells was also observed in transgenic mice expressing biologically active IL-2 on beta cells (RIP-IL-2 mice) [245, 246] No autoimmunity occurred [245]. Crossing RIP-IL-2 transgenic mice with RIP-H-2K^b resulted in double transgenic mice which showed no sign of autoimmune disease despite an impressive early islet infiltration. This suggested that although IL-2 can attract inflammatory cells to the pancreas it could not activate cells specific for islet autoantigen K^b. Thus although IL-2 can break tolerance <u>in vitro</u> it maybe unable to prevent the induction of anergy. Another molecule associated with anergy, B7-1 was also placed under the rat insulin promoter [247]. Autoimmunity resulted when B7-1 transgenic mice were crossed with mice expressing TNFα on the islet cells, suggesting that the B7-1 co-stimulator alone is not sufficient to abrograte tolerance to peripheral antigens.

<u>viii) Peripheral tolerance and superantigens</u>

Peripheral tolerance to superantigens has also been shown in both non-transgenic and transgenic mice. Non-transgenic mice expressing Mls^a and I-E were treated with anti-I-E antibodies and the presence of Mls^a reactive cells; i.e. T cells expressing a TCR utilising Vβ6, were analysed [248]. Vβ6+ve T cells were usually deleted in mice expressing both I-E and the Mls^a antigen however following anti-I-E treatment Vβ6+ve cells were present in both the thymus and periphery. These cells

were not functionally tolerant since they proliferated and produced cytokines following TCR stimulation. Upon cessation of antibody treatment, I-E was re-expressed in the periphery with a concomitant reduction in number of Vβ6+ve cells in both lymph nodes and spleen.

Peripheral deletion in non-transgenic mice was also shown by Webb et al (1990) [249]. In this model transfer of H-2 compatible Mls^a antigen positive lymphoid cells into Mls^b mice resulted in clonal elimination of Mls^a reactive Vβ6+ve cells. However unlike the Jones model deletion was preceded by a powerful in vivo immune response. Activation prior to deletion was also shown by Fink et al (1994) [250] with cell activation markers CD44 (Pgp-1) and VLA-4 being expressed at high levels.

Injection of normal mice with high and low doses of Staphylococcal enterotoxin B (SEB) resulted in anergy of SEB reactive Vβ8+ve T cells [251]. Anergy following injection of either a low or high dose of SEB was short or long-lived respectively. 50% of these anergic Vβ8+ve cells had down regulated their TCR/CD3 complex as well as CD2, CD4 and CD8 accessory molecules [251].

Transgenic mice have also been used to look at the effect of superantigens in the periphery [252-258]. Transgenic mice expressing Vβ8.2 TCR+ve cells (99% of CD4+ve and CD8+ve) plus an endogenous TCRα chain, were injected with SEB. This resulted in a rapid increase in the number of both CD4+Vβ8.2+ [252, 253] and CD8+Vβ8.2+ spleen and peripheral lymph node cells [252]. During this proliferative phase there was an increase in activation markers CD44, LY6A/E and H1.2F3 as well as an increase in IL-2 expression [254]. Following this short burst of activation the absolute number of cells decreased due to either deletion or re-distribution to other tissues [252-254]. However some Vβ8.2+ve T cells remained which were anergic: unable to respond to SEB in vitro and did not produce IL-2 [252]. This unresponsiveness was reversible in the presence of rIL-2 or by crosslinking either the TCR or CD3 with antibodies. Perkins et al (1993) [253] also found that

the CD4+Vβ8.2+ T cells remaining were unresponsive to SEB (although they produced IL-2) and also to rIL-2, IL-4 or IL-6. They were also unresponsive to restimulation with superantigens, ConA, phytohaemagglutinins (PHA) or anti-CD3. Unresponsiveness was only broken if stimulation via cell surface receptors resulted in PKC and Ca^{2+} activation.

Similar results were observed following repeated injection of Staphylococcal enterotoxin A (SEA) into Vβ3+ve transgenic mice [255]. Anergic CD4+Vβ3+cells remaining after proliferation did not respond to rIL-2 and reduced IL-2 and TNFα mRNA levels were detected in these cells. In contrast the number of CD8+Vβ3+ cells, with CTL activity, increased following SEA injection and there was no reduction in IFNγ levels. This cytokine being predominantly released by CD8+ve cells. This study suggested that there was selective tolerisation of CD4+ T cells.

The effect of Mls^a antigens in the periphery was also studied in transgenic mice expressing Vβ8.1+ve transgenic TCR [256-258]. As above, exposure to Mls^a in the periphery either through injection of Mls^a spleen cells, mating onto an Mls^a background or in bone marrow chimaeras, resulted in tolerance to Mls^a in vitro, following a period of proliferation. The remaining cells were anergic, they were found either not to produce IL-2 [258] or produce it in low concentrations [256] and they did not respond to rIL-2 [257]. The mechanism of tolerance was shown to vary depending on how Mls^a was introduced into the recipient [256].

To investigate this mechanism of tolerance, Mls^b (Vβ8.1+ve) mice were injected with Mls^a+ve cells before being grafted with Mls^a skin. These mice showed delayed allograft rejection as compared to uninoculated transgenic mice [257].

Like the superantigen models described above, tolerance in the mature T cell population was found using transgenic mice with T cells specific for LCMV. Injection of soluble LCMV into these mice resulted in tolerance through deletion and anergy of LCMV specific T cells after an initial expansion period [240].

ix) Peripheral tolerance summary

Peripheral tolerance to extrathymically expressed antigens has been described. Mechanisms implicated in peripheral tolerance are similar to those of central tolerance, for example; deletion, anergy and down regulation of CD8/TCR. Deletion of self-reactive peripheral T cells, through apoptosis, may follow a period of activation. Self-reactive T cells, in an anergic state, have been found following this process although in other circumstances anergic T cells have been found independent of deletion. In some experiments anergic T cells can become functional either following in vitro stimulation with rIL-2 or after the removal of antigen in vivo. Although anergy is reversible the presence of antigen ensures that a tolerogenic state persists.

Anergic T cells are susceptible to further tolerogenic signals resulting in; 1) a T cell in a deeper anergic state, which is still reversible or 2) deletion of the cell.

TCR/CD8 down regulation may also occur in the periphery. This process is also reversible following an appropriate signal, for example, rIL-2 or anti-CD2 stimulation.

Other routes of introducing extrathymic antigens have been studied. Soluble antigens have been introduced intravenously, intraperitoneally, nasally and orally. In all these cases tolerance can result. The next section is restricted to a discussion of oral tolerance.

1.7) Oral tolerance

Exogenous administration of an antigen to the peripheral immune system via the gut results in systemic and mucosal tolerance [261-263] at both the antibody and T cell level. This phenonomen is known as oral tolerance. This tolerogenic state is antigen specfic and is influenced by age [264], genetic background [265-267], nutritional status of the animal [268], rate of antigen uptake [264, 269], intervals between feeding [264] and the persistance of antigen [269]. Primed animals cannot be orally tolerised to the priming antigen [270].

Recently, oral administration of an autoantigen has been shown to result in suppression of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) [271-276], uveitis [277], myasthenia gravis [278], collagen and adjuvant induced arthritis and diabetes in NOD mice [279]. Prolonged graft survival and suppressed alloreactivity has also been demonstrated through oral administration of the alloantigen [279].

The above experiments and those looking at the effect of feeding protein antigens such as ovalbumin [269, 270, 280-282], HEL [283, 284] and SEB [285] have provided insight into the mechanisms of oral tolerance induction. Deletion has been ruled out as a possible mechanism since following a single oral dose of protein tolerance lasts for about 60 days before recurrence of a systemic response (following challenge). Strobel and Ferguson (1987) [286] also showed differences in the duration of oral tolerance for antibody and T cell responses: antibody tolerance lasted 3 months whilst T cell tolerance lasted 6 months.

Two mechanisms have been suggested to explain the phenonomen of oral tolerance; suppression (direct or indirect) and anergy, although these mechanisms may not be mutually exclusive. Suppression involves the generation of regulatory cells that suppress an immune response in vitro and in vivo via the secretion of cytokines. On the other hand, anergy describes the unresponsivness of T cells.

The dosage of antigen given orally has been shown to affect which of these mechanisms occurs [277, 284]. A low dose of antigen, for example 5 intragastric feeds of 250-300μgs of experimental autoimmune uveitis (EAU) autoantigen S-antigen or S-antigen peptide [277] resulted in suppression, whilst a high dose of antigen, for example a single 5mg feed resulted in anergy [277]. It is thought that suppression was a result of antigen processing and presentation by gut associated APC's whilst anergy occurred after antigen had passed into the periphery via the gut [279]. The latter occurred rapidly (within a 24 hour period) in the absence of activation,

differentation or proliferation [270, 280]. The mechanism of anergy in this case may have been due to the exposure of a soluble protein to the systemic immune system in the absence of co-stimulation [280]. Confirmation of this was found in OVA feed mice. Soluble OVA was present in the serum of mice 5 and 60 minutes following a 25mgs OVA feed [287]. Injection of serum taken 60 minutes after feeding into a secondary recipient transferred systemic tolerance.

i) Suppression

Early experiments suggested that a suppressor mechanism was induced following oral administration of an antigen [259-261, 266, 288-290]. Feeding of substances thought to inhibit suppressor cells, eg. cyclophosphamide [261], deoxyguanosine [290] and oestradiol [288] resulted in the lack of oral tolerance to OVA at the antibody and cell mediated level as well as inducing a local DTH response similar to that seen during food allergy.

Recent data on the induction of tolerance following autoantigen feeding has confirmed the existence of suppression in vivo and in vitro [265, 273-275, 278]. In these experiments suppression appeared to be antigen specific. For example feeding Lewis rats with nicotinic acetylcholine receptor (ACHR) resulted in suppression of experimental induced myasthenia gravis but these rats developed EAE following parenternal immunisation with myelin basic protein (MBP) [278].

Both CD4+ve [276, 279] and CD8+ve [265, 274, 275, 278, 279] cells have been implicated as the suppressor elements. Whole and CD4-/- depleted spleen cells but not CD8-/- depleted spleen cells from MBP fed Lewis rats suppressed MBP specific responses in vitro, indicating the importance of CD8+ve cell in this disease [265]. CD8+ve T cells were also shown to be the mediators of orally induced suppression in adoptive transfer experiments.

Suppression did not require cell to cell contact. Tolerance occurring through the release of non-antigen specific

cytokines [265, 274, 275]. CD4+ve clones isolated from mice fed MBP secreted TGFβ, IL-4 and IL-10 following MBP stimulation [276]. CD8+ve suppressor cells were also thought to induce tolerance through the secretion of TGFβ [265, 275, 278, 279]. The active form of TGFβ was found in vitro in the supernatants of antigen stimulated T cells from mice orally tolerised to MBP and OVA [275]. The importance of TGFβ was further documented; 1) anti-TGFβ administered in vivo abrograted the effect of oral tolerance to MBP and resulted in EAE in susceptible mice. 2) systemically administered TGFβ (1-5μgs) suppressed the severity of the pathology in animal models of autoimmune diseases such as EAE and arthritis. 3) Peyers' patches cells from mice given antigens orally produce TGFβ and can transfer tolerance. 4) TGFβ has also been found to be elevated in the brains of MBP orally tolerised Lewis rats [272]. After feeding MBP and LPS to Lewis rats EAE pathology was suppressed and the levels of TGFβ, IL-4 and prostaglandin E_2 (PGE_2) produced were increased [272]. However the transfer of serum from orally tolerised animals does not suppress EAE suggesting that these antigen non-specific cytokines may act at a local level [274].

Suppression may also work through a mechanism known as the "bystander effect" [265, 274]. This "bystander effect" was shown using a transwell system in which irradiated spleen cells from mice fed tolerogenic MBP were separated from an OVA specific T cell line. In the presence of MBP the reactivity of the OVA cell line to OVA was inhibited. No suppression was found with PBS fed irradiated spleen cells in the presence of MBP. This "bystander effect" was also seen in vivo. For example feeding rats OVA followed by a OVA+MBP-FCA footpad immunisation resulted in a suppressed immune response to injected MBP.

ii) Anergy

In vitro stimulation of T cells from the Peyers' patches, spleen and mesenteric lymph nodes of OVA fed tolerant mice produced a cytokine profile similar to that found for T cell

clones rendered anergic through exposure to high doses of synthetic peptide or fixed APC's [291]: GMCSF and IFNγ but no IL-2, IL-4 or IL-3 cytokine were found in the supernatants [271, 281]. Melamed and Friedman [270, 280] also demonstrated that anergy occurred following an OVA feed. In vitro culturing of peripheral lymph node cells from tolerant mice with OVA resulted in reduced IL-2 and IFNγ production, whilst IL-4 levels were unaffected compared to saline fed controls. Incubation of these tolerant cells with rIL-2 for 5 days resulted in a slight increases of IL-2 and IFNγ production. Migita and Ochi (1994) [285] demonstrated anergy in mice given 4 feeds of 50mgs of SEB. Spleen cells from these mice were hyporesponsive to SEB in vitro as compared to controls. Cytokine production measured following SEB stimulation indicated that these cells did not produce IL-2. These non-responsive cells did not respond to anti-CD3, non-specific T cell mitogens, rIL-2, ConA or PHA.

Cytokine production was also analysed in peripheral lymph nodes cells from mice fed HEL (20mgs/mouse or 1mg X 5 doses/mouse) [283]. It was found that IL-4 mRNA levels had increased following re-exposure to antigen by immunisation. This was not seen if re-exposure was via feeding. IFNγ mRNA levels were found to be reduced in the lymph nodes and no increase in IL-10 or TGFβ could be detected in these orally tolerant mice. More recently it has been shown that IL-10 is not important for the induction nor the maintenance of oral tolerance [292]. Friedman and Weiner (1994) [284] have also investigated cytokine production following feeding tolerising doses of HEL (5-20mgs/mouse). They found that in the absence of immunisation, spleen cells from HEL tolerised mice secrete IL-4 and TGFβ, without proliferation, however upon antigen stimulation an increase level of IL-4 occurred whilst TGFβ levels remained constant. Anergy was also found when feeding an auto-antigen, MBP [273]. 4 X 5mgs feeds of MBP in the presence of soyabean trypsin inhibitor (STI), resulted in systemic tolerance and suppression of EAE in rats. Transferring PLN or serum from MBP-fed donors into naive

recipients did not induce tolerance. Suppression was also ruled out since encephalitogenic MBP-specific T cell lines stimulated with antigen plus APC transferred into orally tolerant mice induced clinical disease. It was found that there was a 5-10 fold reduction in the number of MBP-specific lymphocytes capable of secreting IL-2 in the lymph nodes of orally tolerant rats. IFNγ was also reduced.

As described, tolerance to OVA in mice following a single 20mg feed was shown by Melamed and Friedman [270, 280]. These authors found a reduction in IgG2a anti-OVA antibodies in tolerant mice following OVA and FCA immunistion although IgG1 was produced at levels similar to control mice. From these data it would appear that there was a decrease in T_H1 dependent responses with decreased IgG2a, IL-2 and IFNγ, suggesting selective T_H1 tolerance following oral administration of an antigen. On the other hand oral administration of an antigen had no effect on T_H2 cells; IgG1 responses were not reduced nor was the level of IL-4 reduced. In some cases it appears that T_H2 cells are primed following feeding which results in an increase in IL-4.

iii) Oral tolerance summary

Oral administration of an antigen can induce a state of systemic tolerance at both the antibody and T cell level. Two mechanisms implicated in this phenonomen are suppression and anergy. Suppression of an immune response is through the generation of regulatory cells, either CD4 or CD8, and through the release of cytokines, for example TGFβ. Anergy appears to affect T_H1 cells preferentially.

1.7) β-lactoglobulin (BLG) transgenic mice

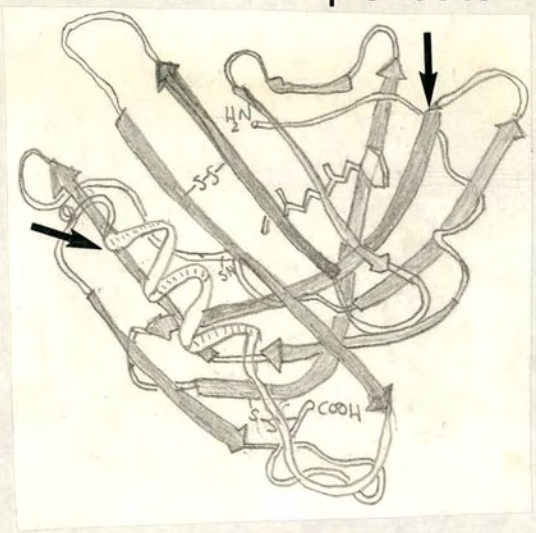
As described above, transgenic mice expressing a foreign protein under the control of either a ubiquitous or tissue specific promoter have been central to recent investigations into the mechanisms underlying both T and B cell tolerance.

Transgenic mice expressing the ovine milk protein BLG in the mammary gland, under the control of its own promoter, have

Fig. 1.5. Drawing of the BLG 3D structure

anti-parallel
β-sheets

α-helix



Adapted from Papiz et al (1986) [**303**]

been produced by Simons et al (1987) [293] and have been used in this study to investigate whether tolerance at the B and T cell level occurred in mice carrying or expressing this mammary-specific gene.

i) β-lactoglobulin

Milk proteins are classed as the acid insoluble, the caseins, or as acid soluble, the whey proteins β-lactoglobulin (BLG) and α-lactalbumin [294, 295]. BLG is present in the milk of cows (where it is the major milk protein at 0.3g/100ml) sheep, deer, pigs, goats, horses, donkeys, camels, buffalos, dolphins and kangaroos [296-300]. Human and rodent milk lacks this protein [301]. BLG is usually present as a dimer which is relatively pH stable, resisting a pH of 2 [302, 303] (thus ensuring that it remains intact until having passed through the stomach [303]). At pH 3.5 BLG dimers dissociate to give 18kDa monomers. At pH 7.5 conformational changes were shown to occur and at pH 9.1 BLG was shown to be denatured [303, 304].
The overall BLG structure was defined using standard crystallographic techniques [303, 305]. It has a high helical content and an unusual folding pattern which is described as a flattened cone, the core consisting of a short α-helix segment and 9 strands of anti-parallel β-sheets which wrap round to form an anti-parallel beta-barrel [303] (see Fig. 1.5). Hydrophilic residues were found on the exterior of this cone, whilst the interior of the 'barrel' is essentially hydrophobic. Comparison of the 3D structure with other proteins, such as Retinol Binding Protein (RBP) has suggested that BLG belongs to a superfamily of proteins with fat-transporting capabilities [297, 303, 306]. Despite their 3D similarities there is limited sequence homology, only 25-30% between the members of this family [297].
RBP binds Vitamin A [307] thus suggesting that BLG maybe important in Vitamin A uptake in the immature gut. This was further confirmed since BLG has been shown to bind Vitamin A [308] and I^{125}-labelled BLG -retinol complexes bound specifically to purified microvilli prepared from the lower

segment of calf small intestine but not to older animal tissue [303]. These data suggested that specific receptors for BLG plus Vitamin A complexes existed in neonatal calves' intestines. The importance of these receptors and subsequent intake of Vitamin A by the newborn animal can be seen in animals deprived of both. Those animals failed to develop, became blind and sterile [297, 309]

Apart from complexing with Vitamin A, BLG has been shown in vitro to bind a variety of hydrophobic substances such as long-chain fatty acids and triglycerides. BLG isolated from cow, sheep and goats milk is associated with between 0.5-0.7 molecules of fatty acids per monomer [300]. This confirmed the idea that BLG was a member of a group of molecules capable of binding hydrophobic ligands as well as having some importance in the delivery of Vitamin A to the young. Vitamin A in milk is associated with fat globules [300].

ii) BLG-transgenic mice.

The BLG-transgenic mice were created to study the effect of manipulating milk proteins: i.e. adding an ovine milk gene into the mouse genome. The ultimate aim was to investigate; 1) whether adding a foreign milk gene resulted in the expression of the product of the gene in milk and 2) what mechanisms were involved in controlling gene expression in mice before manipulating milk protein expression in larger animals such as sheep, pig and cows. Mice were chosen since; a). It was possible to microinject the transgene of interest into a significantly large number of eggs b). The length of pregnancy is relatively short and the time to wait from gene transfer until collecting milk samples is about 3 months as compared to a minimium of 3 years for sheep and c). More progeny are produced.

A gene construct encoding sheep BLG was injected into fertilised eggs as a 16.2 kilobase (kb) insert containing 4kb of DNA 5' to the capsite, the 4.9kb BLG transcription unit and a 7.3kb of 3' flanking sequence or as a fragment with 5.7kb removed from the 3' flanking regions [293]. Regardless of the

DNA sequence, 16 transgenic mice were created carrying 1 to 20 copies of the transgene per cell. Transgenic mice were identified via Southern blotting and probing with a nick-translated p931 BLG cDNA probe [293]. DNA preparations from tails. It was found that 5 of these mice were capable of passing the BLG-transgene to their offspring.

The presence of BLG was confirmed in the milk of lactating transgenic mothers from line 7, 14 and 45 by SDS page gels electrophoresis and Western blotting. The concentration of BLG in the whey was measured by densitometry of Coomassie Blue stained SDS gels using internal BLG controls. The concentration of BLG in the milk of line 45 BLG-transgenic mice, used in this project, was 14.1+/-0.41 to 21.6+/-1.6 mgs/ml-1 of ovine BLG [293].

The expression of the BLG transgene was analysed via RNA extraction and Northern blotting. BLG mRNA was present only in the mammary tissues [293] specifically in the secretory epithelial cells [310] and in virgin transgenic females at very low levels with increasing expression during pregnancy and lactation [310, 311]. When transgenic kidney, liver, spleen, salivary and lachrymal glands were analysed for the presence of BLG mRNA no expression was found [293, 311]. Immunohistochemical analysis indicated that BLG mRNA expression was present in the salivary glands of these transgenic mice, although no protein expression was found [311].

In summary, ovine BLG-transgenic mice carrying the BLG gene, under the control of its own promoter sequences have been made and the mice and offspring are healthy and fertile. The translation of the ovine BLG gene is tissue specific, ie: to the mammary epithelial cells.

The low level of expression in virgin mammary tissue increases throughout pregnancy and lactation such that ovine BLG protein is present in the milk of transgenic mothers at 5 times the concentration of ewes milk [293, 310, 311]. Developmental regulation of BLG in both sheep and mice was shown to be similar [310]. Thus *cis*-acting sequences, which

determine ovine BLG mammary expression, are recognised in an animal which is negative for an equivalent gene [310].

1.8) Aims of the thesis

The aims of the thesis were;

i) To develop a simple method of purification for ovine BLG from sheep's milk.

ii) To develop an enzyme linked immunosorbant detection assay (ELISA) and a DTH assay for ovine BLG.

iii) To investigate whether ovine BLG was immunogenic. Could this protein induce antibody and DTH responses?

iv) To investigate whether possession or expression of the BLG-transgene interfered with the normal immune response to ovine BLG; were mice possessing the BLG transgene tolerant to the product of their gene?

v) To investigate whether expression of the BLG-transgene occurred in the thymus.

vi) To investigate whether ovine BLG was an oral tolerogen when administered during suckling.

vii) To investiagte whether adult animals could be rendered tolerant to bovine BLG following oral administration.

Mice transgenic for the protein ovine BLG are good models for the study of tolerance to a self and foreign protein since;

i) As described in previous sections mice transgenic for various proteins, expressed in the periphery, are tolerant at the T and/or B cell levels. Recent data has suggested that tolerance to peripheral transgenes is due to expression of protein in the thymus. Unlike these models expression of the BLG protein is both temporally controlled and tissue specfic, only being expressed in the lactating mammary gland. This makes them good models for peripheral tolerance.

ii) As most food allergies, for example cow's milk allergy, occurs in childhood this mouse model allows the effect of protein administration throughout postnatal development to be investigated. Unlike other mouse models relatively high

concentrations of protein (BLG), present in transgenic mouse milk, can be delivered orally to newborn mice via a natural suckling mechanism and exposure to this protein continues until weaning. This obviates the need for potentially damaging and traumatic oral routes of immunisation, for example intubation.
As BLG has been implicated as one of the main antigens of milk this model allows further investigation of this protein with respect to its causation of allergic responses in the newborn.

iii) The experiments can be internally controlled since the transgene can be inherited either from the paternal or maternal side, i.e. with or without exposure to transgenic milk.

CHAPTER 2

MATERIALS AND METHODS

All reagents and chemicals supplied by SIGMA (Poole, UK) unless stated.

2.1) Beta-Lactoglobulin (BLG) purification and analyses

i) Source of ewes milk.

Ewes from the laboratories and farms of the BBSRC Roslin Institute, Roslin, Midlothian, were used as the sources of sheep's milk. Following alcohol sterilisation of teats, lactating ewes were milked by hand (Dr. M McClenaghan and staff of the Roslin Institute farms kindly helped with the milking operation). Milk was stored at -70°C until required.

ii) BLG purification

Since ovine BLG was not commercially available the purification method of Armstrong et al (1967) [312] was adopted. The methodology is summarised below. 500 - 1000 mls of milk were used per purification.

264gs of solid $(NH_4)_2 SO_4$ was added to one litre of ewes' milk over a period of 35-45 minutes. The solution was stirred constantly for 2 hours before being suction filtered through Whatman 541 filter paper. The collected supernatant was then re-filtered through Whatman 542 paper and stored overnight at 4°C.

The pH of the solution was adjusted to 3.5 over a period of 40 minutes with 1M HCl, and precipitated proteins removed after a 40 minute centrifugation, 13,200g at 16°C. The remaining supernatant was then adjusted to pH6 using IM NH_3 (BDH Ltd, Poole, UK) and precipitated further with $(NH_4)_2 SO_4$. The precipitated proteins were then pelleted out following centrifugation, as described, and then dissolved in a small quantity of dialysis buffer. The proteins were dialysed against

a 0.12M Sodium Acetate- 0.04M Acetic acid (BDH Ltd, Poole, UK) buffer (pH5.2) for 24 hours with fresh buffer being added after the first 12 hours. The solution was then re-dialysed against distilled H_2O (dH_2O) for a period of 3 days. This resulted in a colloidal solution of fine white crystals which was then collected and stored at -70°C before being freeze dried overnight.

iii) Protein analysis

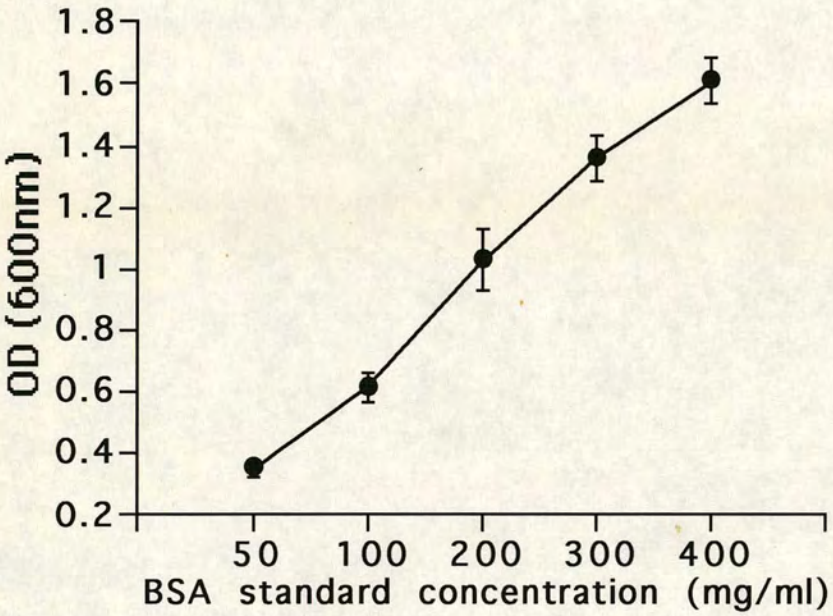
Following purification and freeze drying the protein powder was weighed and the percentage of protein present in the sample was calculated using the Lowry procedure [313-315]. The components of this assay were supplied as a standard protein assay kit which included all reagents necessary to perform the test. These were the protein standard, Bovine Serum Albumin (BSA) at a concentration of 400μgs/ml, the Lowry reagent and the Folin and Ciocalteu's Phenol Reagent all of which were reconstituted as described in the manufacturers instructions. Two types of protein assay could have been performed, the direct (not involving protein precipitation) and the indirect (involving precipitation) assay. The former method was employed in this study. Each step used in the procedure can be summarised as follows;

STEP 1; Both the standard and test samples were diluted to give a range of concentrations as described below (*). 1ml of each dilution was added to appropriately labelled tubes. The test blank was 1ml of H_2O.

(*) A BSA stock solution (400μgs/ml) was prepared to give 5 protein concentrations; 50, 100, 200, 300, 400μgs/ml. Following freeze drying of purified ovine BLG, 10mgs of the resulting precipitate was weighed out and dissolved in 1ml of dH_2O. This BLG "stock" solution was then further diluted to give a concentration range of 62.5, 125, 250, 500, 1000 μgs/ml.

STEP 2; 1ml of Lowry reagent solution was added to each tube and following a vigorous mixing each solution was left at room temperature for 20 minutes.

Fig. 2.1. Standard curve of a range of a BSA standard concentrations for use in a Lowry test.



Each point represents the mean of 5 Lowry tests, +/- 1 standard deviations.

STEP 3; 0.5mls of Folin and Ciocalteu's Phenol Reagent was added to each tube whilst vortexing and the solutions left at room temperature for a further 30 minutes during which time a colour change occurred.
STEP 4; The absorbancy of each tube was measured at a light wavelength of 600nms with the test blank being used as the zero. A plot of the absorbancy of the BSA standard solution versus appropriate protein concentration provided a standard calibration curve from which the protein concentration of test samples was calculated. The mean BSA standard concentrations curve is shown in Fig. 2.1 (raw data shown in Appendix 1).
STEP 5; The protein concentration of the BLG samples was read from the standard curves. The percentage protein in the sample was then calculated using the following equation;

$$\frac{\text{PROTEIN CONC ON X-AXIS}}{\text{CONCENTRATION OF PROTEIN IN TUBE}} \times 100$$

Each of the samples' OD values, were plotted and the concentrations noted. The percentage protein content was then worked out for each of the sample dilution and an average taken. The amount of protein recovered from the purification steps was calculated as;
AVERAGE PROTEIN PERCENTAGE X TOTAL WEIGHT OF SAMPLE RECOVERED.

Of the six BLG purifications performed the total amount of protein recovered from each was as follows;

Purification number	Amount of protein recovered
1	317mgs
2	586mgs
3	710mgs
4	750mgs
5	520mgs

6	420mgs

Differences in the amount of protein recovered may have been due to the point in the lactation cycle from which the milk sample was collected or may reflect the fact that these samples were purified at different times.

iv) Purity testing of the BLG isolates

To prove 1) that the protein isolated from the sheep's milk was indeed BLG, 2) that no other milk protein had contaminated the preparation and 3) that antigenic intergrity was maintained, each of the six preparations of BLG were dissolved in PBS to give 31.7, 58.6, 71, 75, 52 and 42mgs/ml respectively. To test for purity the BLG preparations were subjected to SDS page gel electrophoresis and either stained with Coomassie blue or Western blotted. Antigenic integrity was assessed by Western blotting and ELISA assay.

a) SDS page gels.

All solution recipes can be found in Appendix 2.

A 15% polyacrylamide gel solution was poured into a minigel apparatus (Hoefer Scientific Instruments, UK). The gels were allowed to set over a period of 2 hours and to ensure that no evaporation occurred, as well as maintaining the flat surface of the gels, a small amount of dH_2O was layered on to the top of each gel. Once the SDS gel had solidified the stacking gel solution was added which was then left for 30 minutes to solidify. 10μls of protein sample was diluted in 10μls of dH_2O plus 20μls of sample buffer and each sample was boiled for 2 minutes. 2.5μls of the protein plus buffer mixture was added per well and samples were then run for 45 minutes at 20mA per gel.

Following electrophoresis, gels were carefully removed from the minigel apparatus and then stained overnight in Commassie Blue stain. Following destaining gels were dried under suction.

Figure 2.2 shows the Commassie Blue staining profile of the six BLG purifications. From the gel profiles it was observed

Fig. 2.2.

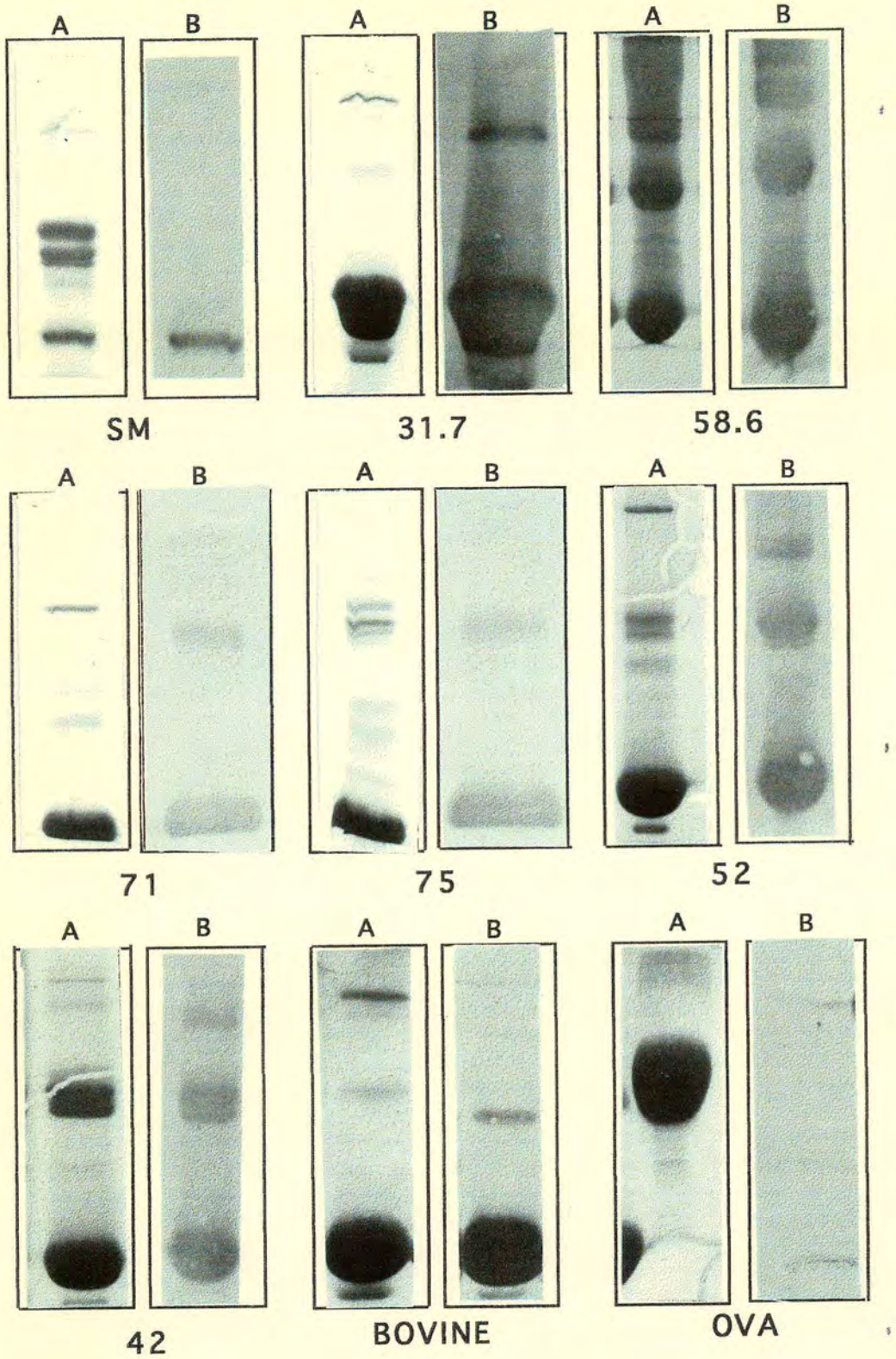


Fig. 2.2. SDS-page gels (A) and Western Blots (B) of 6 BLG purifications from different batches of sheeps milk.

Pairs of SDS gels (A) and Western Blots (B) are shown for 6 ovine BLG purifications which yielded 31.7, 58.6, 71, 75, 52 and 42mgs of BLG/ml. The first pair of photographs labelled (SM) represent a SDS gel and Western Blot of unpurified whole sheeps' milk. The pairs of photographs labelled BOVINE and OVA are SDS gels and Western Blots from commercially purified bovine BLG (SIGMA, Poole, UK) and Ovalbumin (SIGMA).
SDS gels were stained with a Coomassie Blue dye and Western Blots were stained with a rabbit (Rabbit 669) anti-ovine BLG antiserum and an anti-rabbit HRP (SAPU, Carluke, UK) and developed with H_2O_2 (SIGMA).

that most of the samples contained, in addition to BLG, many other protein bands. This may be a reflection of the stage in the lactation cycle from which the milk sample was taken or may reflect inefficiencies in the protein purification procedures.

b) Western blotting.

Western blots were performed following SDS Page gel electrophoresis to check the purity of each protein sample as well as to test whether BLG remained antigenically intact after the purification procedure.

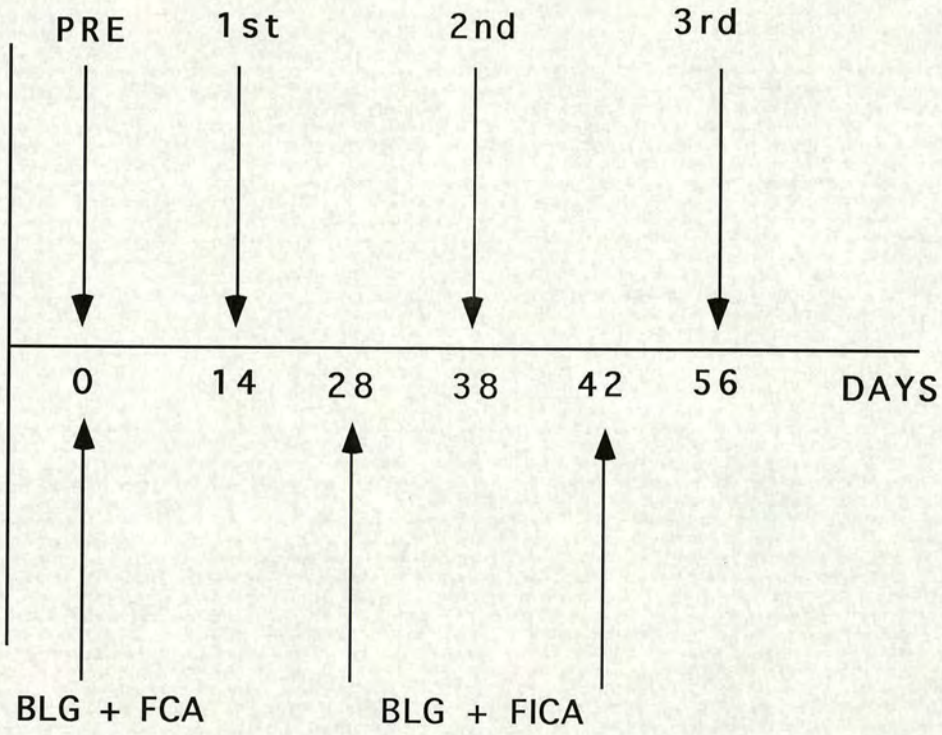
Sheep's milk and the newly purified protein samples were electrophoresed on a 15% SDS page gel, as described above, and afterwards the proteins were transferred from the gel onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) by electrical transfer, 5mAmps, overnight with transfering/blotting buffer.

The membrane was then blocked for one hour, with a PBS solution containing 5% BSA plus Tween 20, before the first antibody was added; an anti-ovine BLG IgG, raised in a New Zealand Black rabbit 669 (see Fig. 2.3). Rabbit 669 serum was diluted (1/100) in blocking buffer before being added to filters. After overnight incubation, with gentle constant agitation, excess anti-ovine BLG antibodies were removed by washing the filter 3 times in PBS-Tween. Horse radish peroxidase (HRP) enzyme linked second step antibodies (donkey anti-rabbit IgG; Scottish Antibody Production Unit (SAPU), Carluke, UK), were then added to the filters at a 1/500 dilution in PBS-Tween. Again filters were incubated overnight with constant agitation.

Unbound, excess antibodies were removed by washing the filter 3 times with PBS-Tween. Before the substrate, 4-Chloro-1 Naphthol, was added filters were washed with a Tris-saline solution. A 4-Chloro-1 Napthol tablet, 30mgs/tablets, was dissolved in 23.3mls of methanol (BDH Ltd, Poole, UK) and 10mls of this solution plus 50mls of Tris-saline buffer was added to each filter. 15μls of H_2O_2 was then

Fig. 2.3 Injection and bleeding protocol for measuring Rabbit anti-ovine BLG IgG responses

BLEEDING SCHEME



INJECTION SCHEME

A New Zealand Black rabbit (Rabbit 669) was immunised with purified ovine BLG (J-M Michard, J D Ansell and S Micklem, unpublished data) plus Freunds Complete Adjuvant (FCA) (PIERCE). Secondary immunisations were performed with Freunds Incomplete Adjuvant (FICA)(PIERCE).
200μgs of BLG was made up in 1ml PBS and to this 1ml of adjuvant was added. The mixture was emulisified before 2mls of Tween 80 were added and the mixture re-emulsified. Rabbit 669 was immunised with 2mls of the resulting mixture.
Rabbit 669 was bled following each of the three immunisations and the anti-BLG titres analysed using ELISA. Serum taken after the third immunisation was used in both Western blot and ELISA assays. Normal rabbit sera was derived from the prebleed sera.

added. If BLG was present on the filter and had been detected by the BLG-specific antibodies, black insoluble precipitate bands appeared.

Figure 2.2 describes the purity of the BLG purification samples. Following Western blotting it was observed that each purified protein sample contained BLG. Rabbit anti-ovine BLG recognises two bands, the low molecular weight band (probably 18kDa BLG momomers) and a higher molecular weight band. No molecular weight markers were included in these gels so the molecular weight of this band is unknown. It is possible that it represents the 36kDa dimer form of BLG. Both these bands were present in sheep's milk.

From the Western blot data it appeared that each of the BLG purifications contained one or more contaminating milk proteins which were not recognised by anti-ovine BLG antibodies. Samples 71, 75, and 58mgs/ml appeared to contain the most contaminants whilst samples 31.7, 52 and 42mgs the least. Although each BLG purification had protein contamination to differing degrees the vast majority of protein purified was BLG.

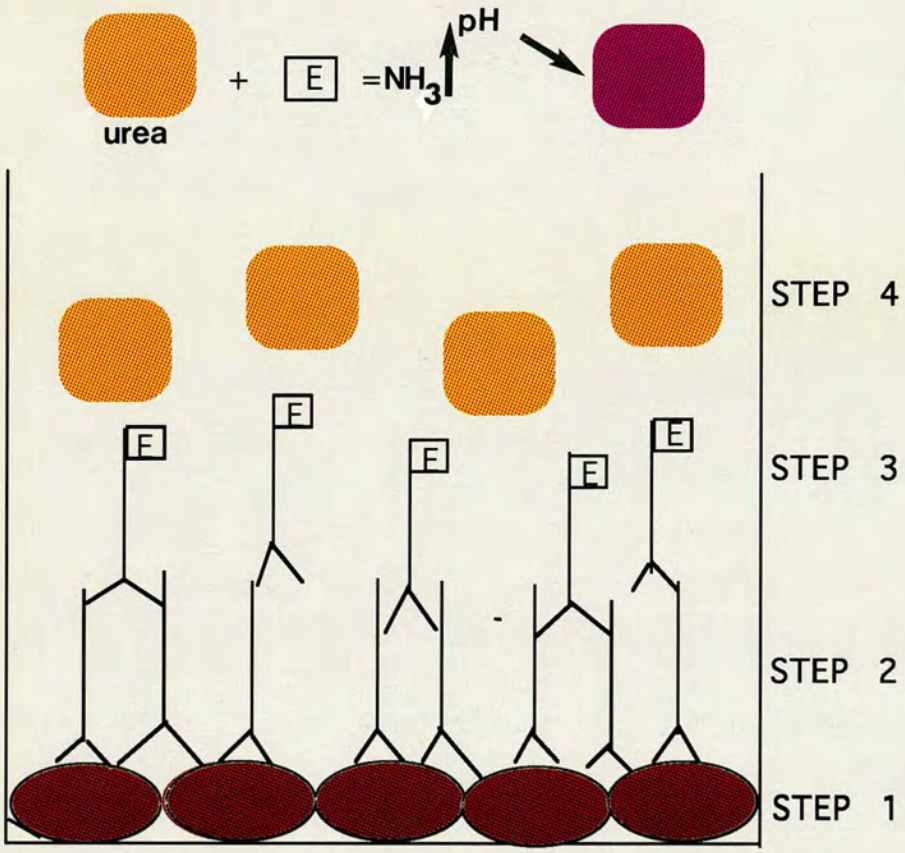
The Western blot data also confirmed the specificity of the rabbit anti-ovine BLG antibody. Bovine BLG was recognised by the rabbit anti-ovine BLG antibodies whilst OVA was not. It was interesting to note that the anti-ovine BLG antibodies also recognised two bands of low and high molecular weights in bovine BLG, suggesting also that perhaps this was the 18kDa momomer and 36kDa dimer form of BLG. It was also noted that bovine BLG (SIGMA) also contained contaminating high molecular weight proteins.

v) Enzyme linked immunosorbent assay (ELISA).

Solutions used in the ELISA system are described in Appendix 3.

Although newly purified BLG samples were recognised on a cellulose filter by anti-ovine BLG antibodies it was important to further investigate protein recognition, again with antibodies, when the antigen was presented on a plastic solid

Fig. 2.4a Cartoon of an ELISA well



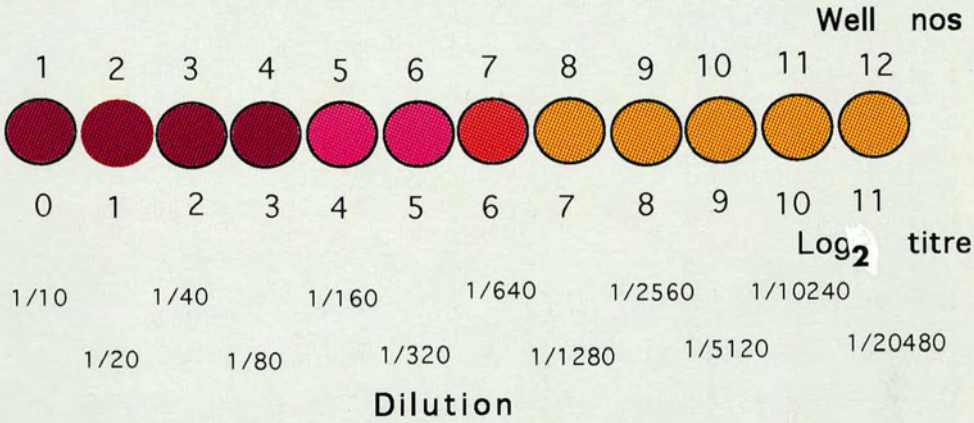
STEP 1 = Coating plate with antigen followed by the blocking antigen.

STEP 2 = Dilutions of sera

STEP 3 = Anti-IgG Urease conjugated antibody

STEP 4 = Urease substrate

Fig. 2.4b. Representation of a typical end point dilution assay



In this example the endpoint is well nos 7, dilution 1/640, titre expressed as 6 Log 2·

phase. An ELISA technique was developed based on a urease detection system [316]. Figs 2.4a and 2.4b describe the principle of the ELISA and the titration system used in this particular assay.

The ELISA protocol used was as follows;

1) BLG samples were diluted in coating buffer at a concentration of 20μgs/ml. This protein concentration was chosen since coating ELISA plates with 20, 50 or 100μgs gave similar OD values for each dilution point of Rabbit 669 anti-ovine BLG sera, see Figs 2.5, 2.6, 2.7 and 2.8. Thus to conserve stocks of protein the lower concentration of 20μgs was chosen.

2) 100μls of the antigen dilution was then added to each well of a 96 well flat bottomed plastic ELISA plate (IMMUNOL 2, DYNATECH, Highwycombe, UK) and plates were incubated for 2 hours at 37°C.

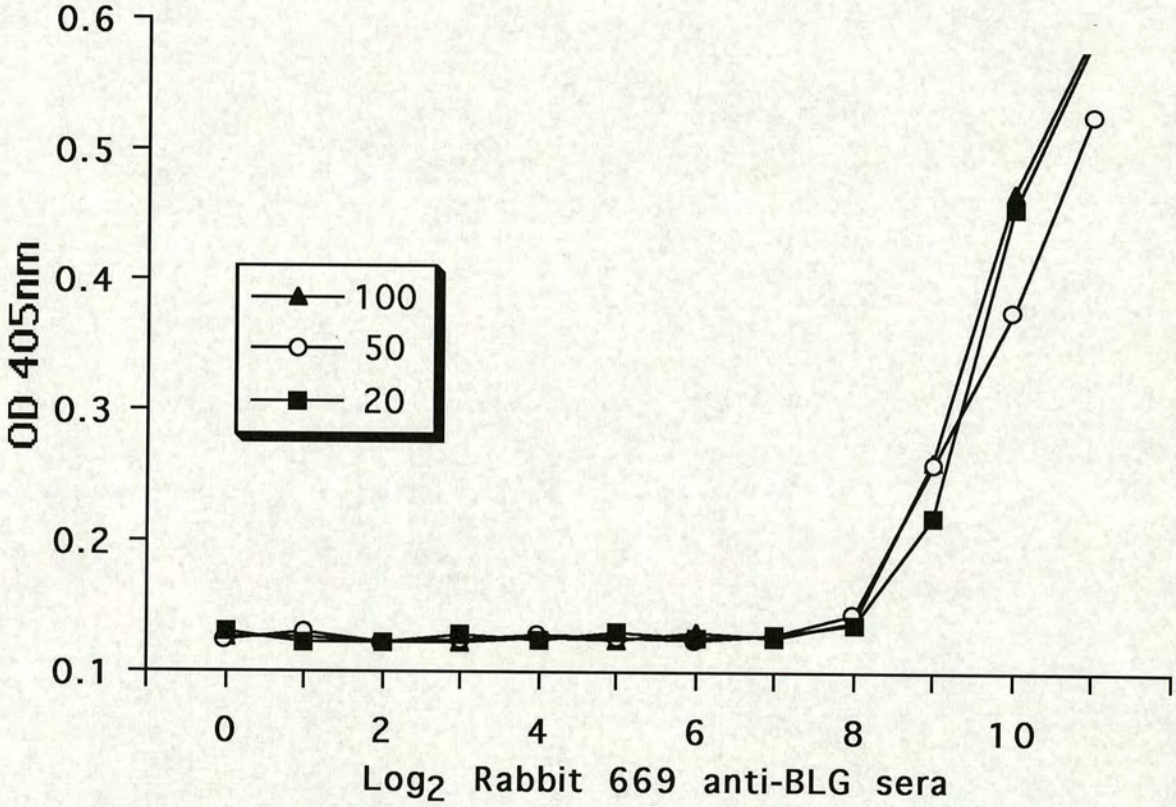
3) After incubation, unbound excess antigen was discarded and plates washed 3 times in wash buffer (WBF) then patted dry.

4) 100μls of diluting buffer (DBF) was then added per well and the plates reincubated at 37°C for 30 minutes. This step ensured that any available binding sites in each well were coated with BSA present in the DBF.

5) Plates were washed 3 times as before with WBF and various dilutions of rabbit anti-ovine BLG sera (Rabbit 669) were then added. Rabbit sera was firstly diluted 1/10 (20μls of sera + 180μls of DBF) then doubling dilutions were made; 100μls of the 1/10 dilution + 100μls of DBF=1/20 and so forth) to give a sera dilution range of 1/10-1/20480. 100μls of each sera dilution were added to the appropriate wells, 1/10 dilution into well 1, 1/20 dilution into well 2 and so on. Triplicates of each sera dilution was made. Plates were then incubated for 30 minutes at 37°C. Control wells contained normal rabbit sera diluted as described above.

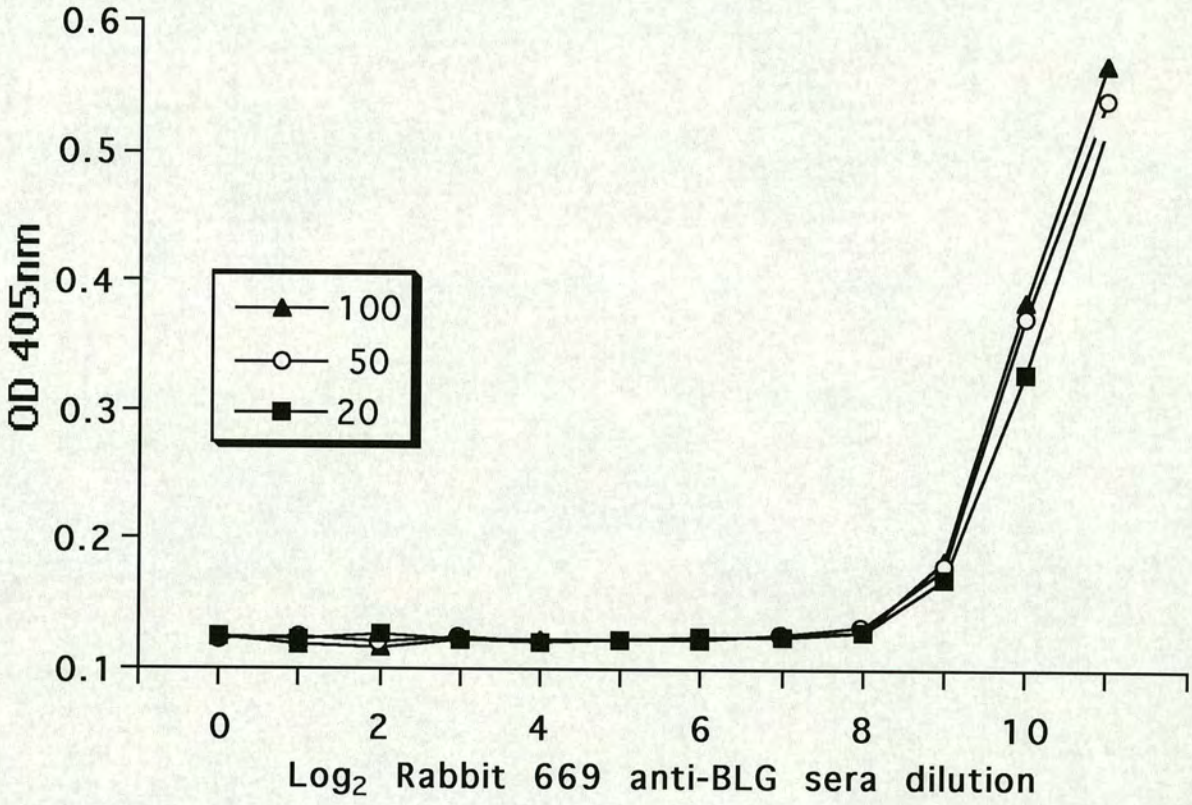
6) Again plates were washed with WBF 3 times. 100μls of a 1/100 dilution of urease coupled anti-rabbit IgG antibodies (SERA-LAB, Sussex, UK) were added per well and plates incubated for 30 minutes at 37°C.

Fig. 2.5. Determination of optimum concentration of ovine BLG with which to coat ELISA wells.



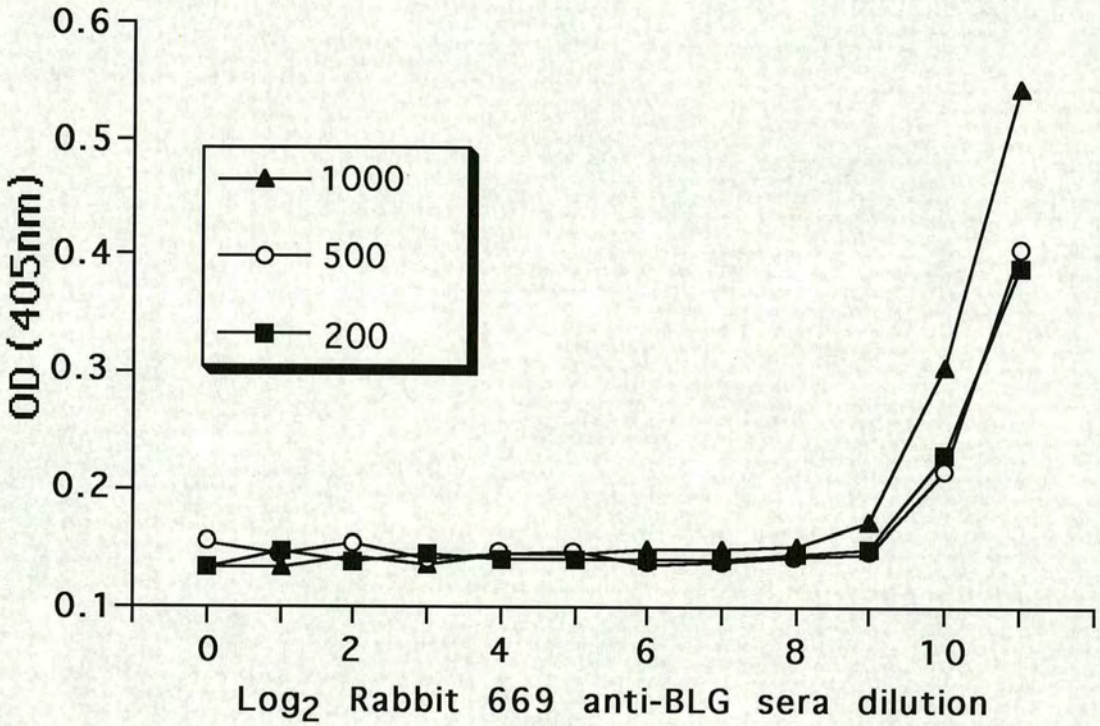
Wells were coated with either 200, 500 or 1000 μgs/ml of ovine BLG from the '31mg' purification batch.
For all coating concentrations the endpoint dilution for binding of Rabbit anti-BLG serum was 9.

Fig. 2.6. Determination of the optimium concentration of ovine BLG with which to coat ELISA wells.



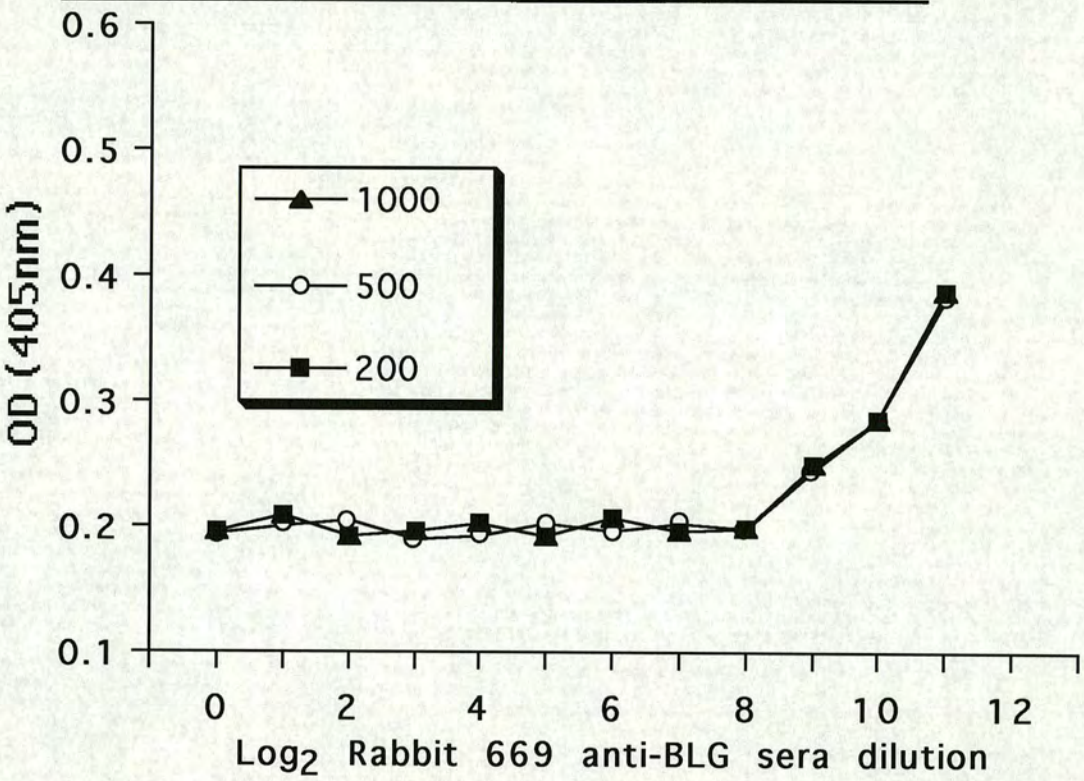
Wells were coated with either 200, 500 or 1000 μgs/ml of ovine BLG from the '58mg' purification batch.
For all coating concentrations the endpoint dilution for binding of Rabbit anti-BLG serum was 9.

Fig. 2.7. Determination of optimium concentration of ovine BLG with which to coat ELISA wells.



Wells were coated with either 200, 500 and 1000 μg/ml of ovine BLG from the '71mg' purification batch.
For all coating concentrations the endpoint dilution for binding of Rabbit anti-BLG serum was 9.

Fig. 2.8. Determination of optimium concentration of ovine BLG with which to coat ELISA wells.



Wells were coated with either 200, 500 or 1000 μgs/ml of ovine BLG from the '75mg' purification batch.
For all coating concentrations the endpoint dilution for binding of Rabbit anti-BLG serum was 9.

7) After washing with WBF, the plates were next washed with dH_2O to remove residual WBF which interferes with the pH of the substrate thus causing false positives.
8) 100μls of substrate was then added to each well. The substate used was urea (SERA-LAB, Sussex, UK) and the system works as follows; the urease coupled to the second step antibody converts urea in the substrate to ammonia, this increases the pH of the solution, which causes a change in the colour of the bromocresol purple, also in the substrate, from yellow to purple.
The substrate was incubated on the plates for 20 minutes again at 37°C. Plates were read at the 405nm wavelength using an automatic ELISA reader. The mean OD value for the triplicates of each dilution was calculated and plotted against a Log_2 transformation of each dilution, for example 1/10 dilution =0, 1/20 dilution=1, 1/40 dilution=2 .... .
As the dilution of the rabbit sera increased the colour change gradually went from purple - red - yellow and correspondingly the OD value increased with this change. In this system the lower the OD value the more purple the solution, the higher dilution the more yellow the solution.
Using the urease detection system in an ELISA allowed a visual approach to working out the antibody titre. In this case the titre would be the reciprocal of the dilution end point and the dilution end point would be the well with the last purple/red colour before a yellow well. For example if a well containing a 1/640 dilution contained a purple/red colour and the next well, 1/1280 was yellow the dilution end point would be 1/640. The titre is the reciprocal of the dilution and in this example the titre is 640, its log_2 transformation is a titre of 6 (see Fig. 2.4b).

Given that the supply of sheep's milk was limited and intermittent it was important to demonstrate that different batches of purified BLG gave similar end points in the above ELISA for a given sera. An ELISA test for each of the six BLG purifications was performed and each BLG sample coated on

Table 2.1. Summary of ELISA assays of Rabbit 669 anti-ovine BLG against various BLG purifications

BLG purification(nos)	Rabbit anti-BLG titre	Rabbit NRS titre
31mgs/ml	>11	(-)
58mgs/ml	>11	(-)
71mgs/ml	>11	1
75mgs/ml	>11	(-)
52mgs/ml	ND	ND
42mgs/ml	>11	(-)

the ELISA plates was recognised by the anti-ovine BLG Rabbit 669 sera. The anti-ovine BLG titres are shown in Table 2.1.
From this data it was concluded that following purification, ovine BLG retained its antigenic properties, and that coating this antigen to plastic ELISA plates allows correct orientation of the protein. Figure 2.9 shows that rabbit anti-ovine BLG sera recognises each purification batch of ovine BLG whilst normal rabbit sera does not (Fig. 2.10).

2.2) MOLECULAR BIOLOGICAL TECHNIQUES

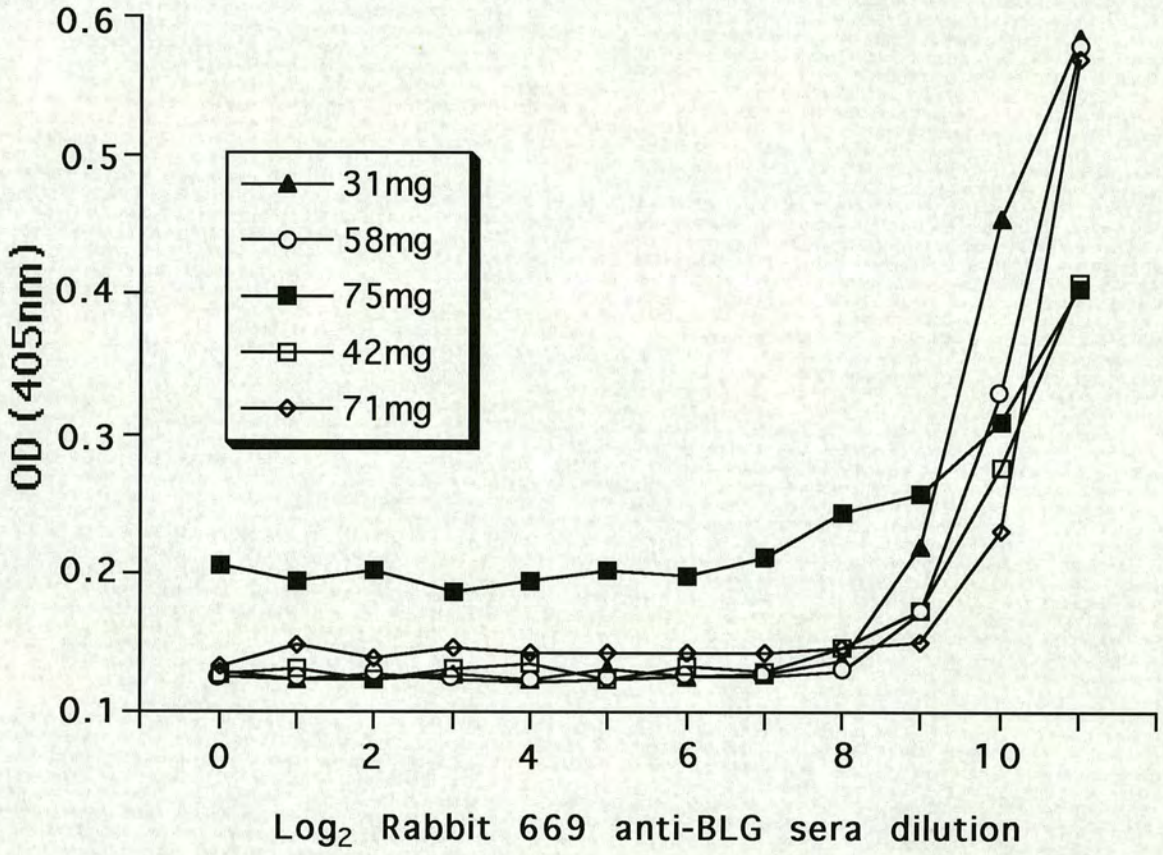
i) Polymerase Chain Reaction (PCR)

For all PCR solution recipes see Appendix 4 .

a) DNA extraction.

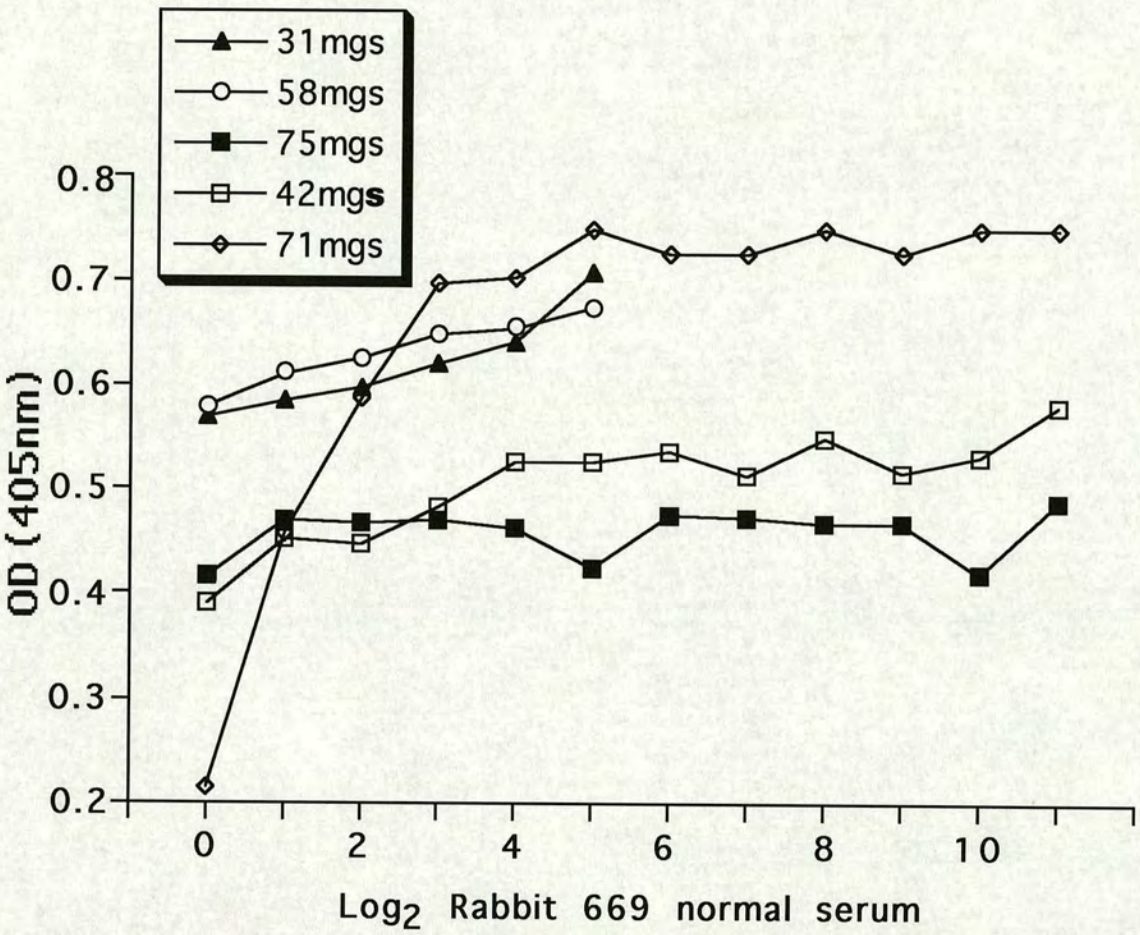
BLG-transgenic and non-transgenic mice were identified via a PCR which amplified sections of transgenic DNA.
Two methods for the extraction of DNA were employed: i) Proteinase K digestion of tail biopses [317] or ii) isolation from peripheral blood [318].
1) Following halothane anaesthesia, using a heat sterilised scalpel blade, 1-2 cm of tail were removed. Each tail section was then cut into 3 smaller pieces before being placed into a screw capped Eppendorff (Corning, Bibby Sterilin Ltd, Staffordshire, UK). 0.5mls of tail buffer plus Proteinase K were then added to each tube and samples were digested at 37°C. Once the tail had been completely digested samples were stored at -20°C until used.
Tail samples were thawed on ice, vortexed and then centrifuged in the cold to pellet out SDS from the tail buffer which can interfere with the PCR reaction.
2) Following halothane anaesthesia, 3 drops of blood from the retro-orbital sinus were collected via a non-heparinised capillary tube (Hawksley and sons Ltd, Sussex, UK) into a sterile Falcon tube (Corning, Bibby Sterilin Ltd, Staffordshire, UK) containing 10mls of red cell lysate. Tubes were inverted to mix the red blood cells with the buffer thus allowing RBC lysis and tubes were left at room temperature for 10 minutes. After centrifuging for 10 minutes at 400g, supernatants were

Fig. 2.9. Comparison of ovine BLG from different purification batchs against Rabbit 669 anti-BLG serum.



All wells coated with 200 µgs/ml of different BLG purifications. In all cases except the '75mg' purification the end point dilution was 9.

Fig. 2.10. Comparison of ovine BLG from differnt purification batches against normal Rabbit 669 serum.



All wells coated with 200 μgs/ml of different BLG purifications. No specific binding of normal rabbit serum was detected with the exception of purification '71mgs".

discarded and the pellet, consisting of some red (RBC) and white (WBC) blood cells, resuspended in 1ml of sterile PBS. This suspension was then transferred to a 1.5ml sterile Eppendorff tube. After microcentrifugation the supernatant was decanted and the remaining pellet resuspended in 200μls of a 1g/ml solution of Chelex 100 (Bio-rad laboratories Ltd,, Hertfordshire, UK) (an $Fe^{2}+$ chelating agent) in sterile PBS. The samples were then heated (by microwaving) for 30 seconds and during this time any $Fe^{2}+$, from the RBC haemoglobin, remaining in the sample, was absorbed by the Chelex and DNA was liberated from the remaining white blood cells. After heating the samples were cooled at 4^{o}C before being centrifuged for 30 seconds, using a microcentrifuge. The remaining supernatants, containing DNA, were used for the PCR.

b) PCR method

1) Primers

Two primers were used, 5' BLG oligo primer which amplifies a 246bp segment of the 5' end of the BLG gene.

5' primer: 5' gct tct ggg gtc tac cag gaa c 3'

3' primer: 5' tcg tgc ttc tga gct ctg cag 3'

These primers were diluted from stock to give 20μM and equal volumes of these 20μM oligos were mixed to give a 5' BLG oligo mix. The final concentration of primer used was 0.1μM (0.5μl per PCR).

The second was the control primer mix which amplified a 332bp segment of the Hypoxanthine Guanine PhospoRibosyl Transferase (HPRT) gene. The enzyme HPRT is ubiquitously expressed in all cells and thus serves as a DNA control. In DNA samples from non-transgenic animals a PCR band corresponding to HPRT should always be present if there is DNA in the tube.

5' primer: 5' gag ttc cgg aac tgc ctt tgg tg 3'

3' primer: 5' ctg tgc cac cgg gcg cat gg 3'

These primers were diluted from stock to 20μM and equal volumes of 20μM oligos were mixed to give a control oligo mix. The final concentration of primer used was 0.1μM (0.5μl per PCR).

2) DNA amplification

Once DNA had been isolated it was PCR amplified. 1μl or 10μls of DNA isolated from tail samples or blood, respectively, were pipetted into an Eppendorff containing mineral oil and denatured for 10 minutes at 95°C. 50μls of the following PCR mixture was then added to each tube;

For each sample:-	5 μls	10xPCR buffer
	5 μls	DMSO
	0.5 μls	BLG primer mix
	0.5 μls	HPRT control primer mix
	0.25μls	Taq DNA polymerase (Boehringer Mannheim Biochemica, East Sussex, UK)
	38.75μls	dH_2O

(A small excess of this mixture was made each time to compensate for pipetting losses and the pipette tip was changed for each tube to avoid cross contamination.)

Controls were included; DNA extracted from the tail of a known BLG-transgenic mouse (from the offspring of a homozygous BLG-transgenic male x CBA/Ca cross), DNA extracted from the tail of a known non-transgenic mouse (CBA/Ca) as well as tube containing no DNA.

Once the PCR mixtures had been added DNA was amplified using a 30 cycle PCR program:-

94°C for 1 minute, the denaturing step
65°C for 5 minutes, the annealling and extension steps.

At the end of the PCR programme the amplified DNA solution was removed into another labelled Eppendorff tube and 12.5μls

Fig. 2.11 : Picture of a PCR gel

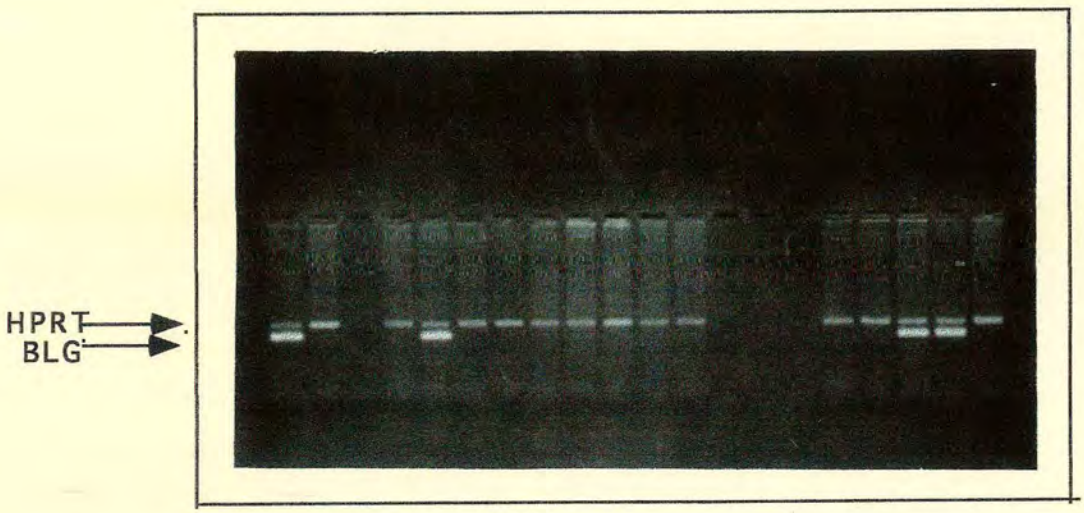


Figure 2.11 represents a picture of an ethidium bromide stained gel containing amplified PCR reactions performed on DNA extracted from digested mouse tail tissue. HPRT and BLG primers, added simultanously, were used in this reaction. Lanes 1, 2 and 3 are control tail DNA samples containing amplified DNA from known BLG transgenic mouse, a normal CBA/Ca mouse and a sample containing no DNA respectively. Lanes 4-20 contained DNA samples from mice of unknown genotype.
BLG positive mice are marked with an *.

of loading buffer was added to each DNA sample. Following mixing,12.5μls of this suspension was loaded onto a 2% agarose gel containing 0.5μgs of ethidium bromide and DNA samples were electrophoresed in TAE running buffer for 30 minutes at 80volts. BLG and HPRT DNA bands present in the gel were then observed following UV illumination. Figure 2.11 represents a typical PCR gel showing both the HPRT and BLG DNA bands.

ii) RNA ANALYSIS

For all RNA solution recipes see Appendix 5.

a) RNA extraction

RNA extraction was performed using RNAzol B solution (Biogenesis Ltd, Bournemouth, UK). This preparation promotes formation of complexes of RNA with guanidinium and water whilst DNA and protein are removed in an aqueous phase. The procedure is very rapid, usually taking less than 3 hours, and is very simple.

Tissues of interest were removed sterilely, to minimise any RNAase contamination, and homogenised with RNAzol B, 2mls of this solution being added per 100mgs of tissue. The homogenate was transferred to a screw capped tube and 100μls of Chloroform (BDH Ltd, Poole, UK) was added per 1ml of homogenate. This mixture was then vigorously mixed for 15 seconds before the tubes were placed on ice for 5 minutes. During this period the RNA is extracted. Following microcentrifugation, at 12000gs at 4°C for 15 minutes, RNA found in the upper aqueous phase of the resulting supernatant was removed into a new tube (DNA and protein being left in the interface and lower aqueous phase). RNA was then precipitated with an equal volume to volume of propan-2-ol (BDH Ltd, Poole, UK) for 15 minutes at 4°C and following centrifugation, 12,000gs at 4°C for 15 minutes, RNA was observed as a white-yellow pellet. After decanting the remaining supernatant, the RNA pellet was washed in 1ml of 75% ethanol (BDH Ltd, Poole, UK) by vortexing and subsequently centrifuged (7500gs at room temperature, for 8 minutes). The RNA pellet

was air dried before being dissolved in diethyl pyrocarbonate (DEPC)-treated 1mM EDTA, pH7 (this step required heating for 10-15 minutes at 60°C) before the concentration of RNA was calculated. The amount of RNA was calculated from the OD_{260nm} value of the sample. RNA samples were stored at -20°C until used.

b) Electrophoresis of RNA samples

RNA samples were run on a 1% formaldyhde reducing gels containing ethidium bromide. 5μgs of RNA was dissolved in 12μls of sample buffer by heating for 5 minutes at 60°C and after cooling, 2μls of staining buffer, bromophenol blue (0.1mg/ml), was added. Gels were run for 2 hours at 100V and destained in several changes of DEPC sterile dH_2O. A photograph was taken before a Northern blot transfer was set up to show the presence of the 18S and 28S RNA bands. Alongside experimental samples an RNA molecular weight marker was also included.

c) Northern blotting

Following destaining of the above gels RNA bands were Northern blotted by capillary transfer onto a Hybond N filter (Amersham International Plc, Buckinghamshire, UK) using the transferring buffer, 20xSSC. Capillary transfer occurred over a period of 18 hours and at the end of this period filters were washed, briefly in 2xSSC buffer, and RNA was fixed onto the filter by baking at 80°C for 2 hours.

d) Probe labelling

BLG mRNA was detected using a ^{32}P labelled BLG cDNA probe [293]. 30ngs of unlabelled probe were denatured for 10 minutes and the following reagents added:-

2μls of Hexanucleotide mix (Boeringer Mannheim Biochemica,, East Sussex, UK)
3μls of dATP, dGTP, dTTP mix (Boeringer Mannheim Biochemica East Sussex, UK)

5μls of dCT^{32}P
1μl of Klenow enzyme (Boeringer Mannheim Biochemica, East Sussex, UK)

^{32}P labelling occurred over a 2 hour period at 37°C. At the end of this period 80μls of TE buffer was added and 2μls of the sample removed to estimate the ^{32}P incorporation. The remaining labelled probe solution was then spun over a G50 Sephadex column to remove any remaining unlabelled probe. Before the ^{32}P BLG cDNA probe was added to the filters it was denatured with NaOH (final concentration of 0.15M)

e) Filter hybridisation

Filters were placed in hybridisation containers before being incubated, at 65°C in a hybridisation oven (Hybaid, Uxbridge, UK), with 25mls of a pre-hybridisation solution. This solution contained 5xSSC, 5x Denhardts' solution, 0.5% (w/v) SDS and 0.5mls of a 1mg/ml solution of sonicated non-homologous spermwhale DNA which had been heated at 100°C for 5 minutes and cooled prior to addition. After an hour the labelled probe was added to the hybridisation containers containing the pre-hybridisation solution and filters. Probe hybridistion took place over a period of 12 hours at 65°C, in a hybidisation oven. At the end of the hybridisation process filters were washed to reduce non-specific binding of the probe. The washing process was as follows:-

2xSSC plus 0.1% (w/V) SDS at 65°C for 10 minutes. Repeat.
1XSSC " " " " " " " . Repeat.
0.1xSSC " " " " " " " . Repeat.

Washed filters were wrapped in Saran-Wrap before being sandwiched between 2 sheets of autoradiograph paper (Kodak Ltd, London, UK) in cassettes. Filters were stored at -70°C for 24 hours before one of the autoradiograph paper was developed. The second was developed a week later for further detection of weak signals.

2.3) Experimental mice

Mice were bred and maintained under standard conditions in the Ashworth Laboratories animal facility and either fed on a maintenance diet (SDS special diets Ltd, Essex, UK) containing whey protein (Appendix 6) or on a whey deficient feed (Harlan OLAC Ltd, Bicester, UK) (Appendix 7).

Breeding stocks were maintained on a breeding diet (Appendix 6). However breeding stocks were maintained on a whey deficient diet when the effect of whey protein in the diet was analysed.

i) Control mice

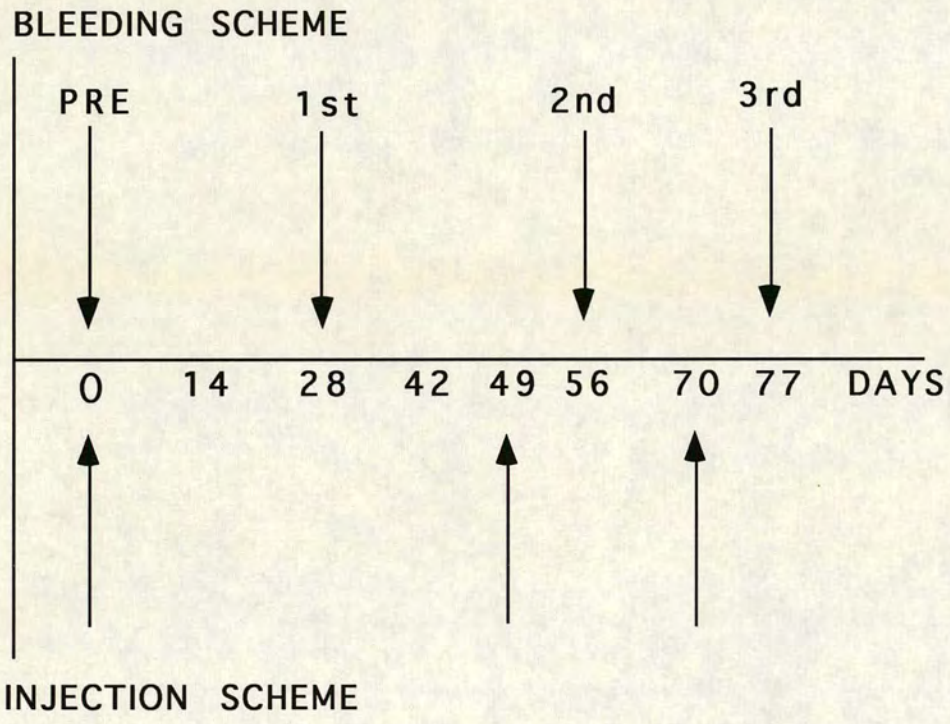
Control mice used were male and female CBA/Ca (H-2^k),. C57BL/6 (H-2^b), CBA x C57BL/6 F1 (H-2^{kxb}) and BALB/c (H-2^d) mice were used to investigate the effect of MHC background on anti-BLG responses. For both antibody and T cell responses mice were immunised at 8-12 weeks of age although some of the exbreeder females were about 20 weeks old at the time of immunisation.

ii) Transgenic mice

Transgenic mice used in my research were transgenic for the ovine BLG gene. These transgenic mice were created by injecting a 16.2 kilobase clone of the sheep BLG gene into fertilised CBA x C57BL/6 mouse eggs. This resulted in three mouse lines, 7, 14 and 45, which were capable of passing the BLG transgene onto their offspring [293]. Line 45 was chosen as the founder line for this project since heterozygous female derived from line 45 transgenic founder mice produced 14.1-21.6mgs of ovine BLG in their milk during lactation [293]. All this work was done by Drs John Clark and Paul Simons group at the BBSRC Roslin Institute, Roslin, Midlothian.

Line 45 homozygous males were crossed with CBA/Ca females and the resulting heterozygous BLG-transgenic offspring were tested for both antibody and T cell responses to ovine BLG. These F1 mice were also crossed with CBA/Ca males or

Fig. 2.12. Injection and bleeding protocol for measuring antibody responses



Mice were injected (ip) with BLG + ALUM on the days specified and were bled for antibody responses as described.

females (F1 BLG-transgenic male X CBA/Ca female or CBA/Ca male X F1 BLG-transgenic female) and the transgenic status of the resulting offspring, either BLG-transgenic or non-transgenic was determined by PCR using BLG primers (see section 2.2). These animals were also tested for both antibody and T cell responses to ovine BLG.

2.4) Immunisation protocols and testing for response to BLG

i) Antibody

a) Immunisation

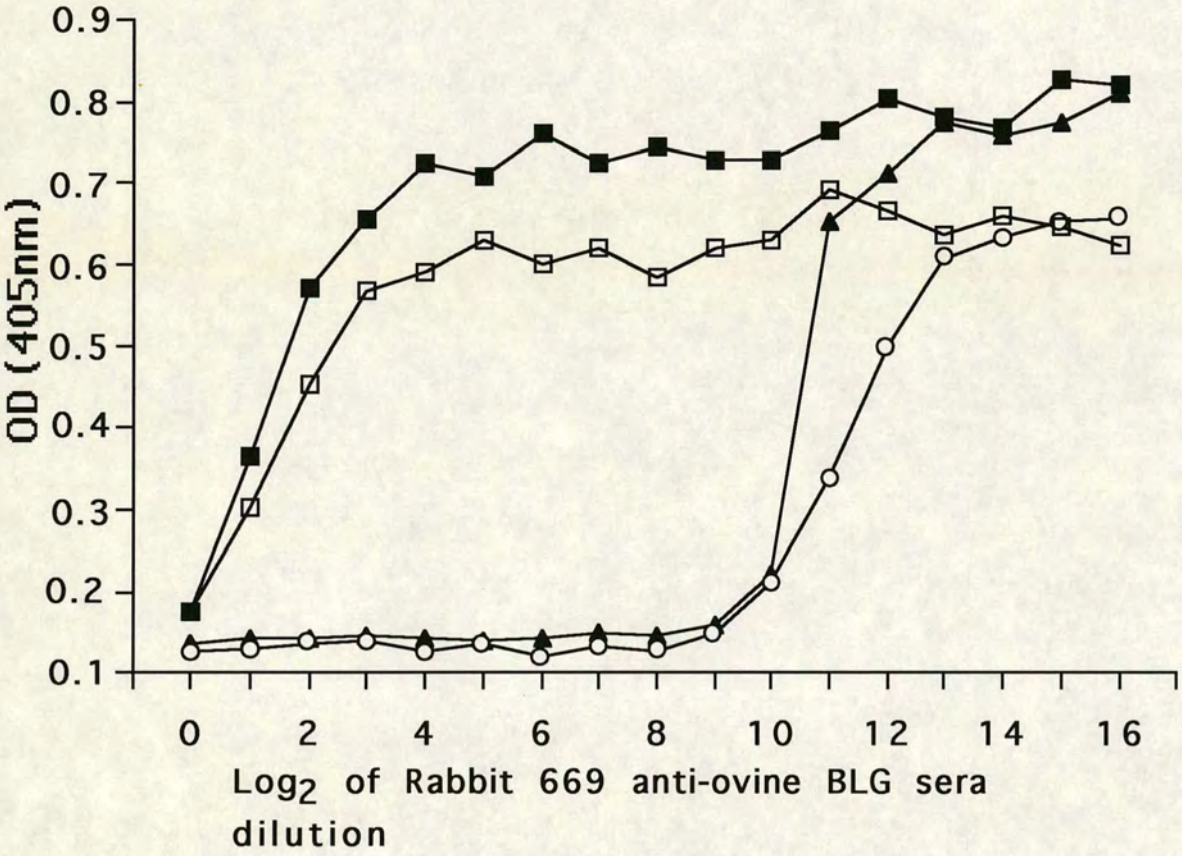
At the beginning of an experiment, mice were randomly allocated to specific experimental groups and from each of these groups a few were bled for sera (pre-bleed). Each mouse was immunised intraperitoneally (ip), using a 21G needle (Becton Dickinson UK Ltd, Oxford, UK) with a total volume of 0.4mls of antigen (50μls in 0.32mls of PBS) with an aluminum hydroxide adjuvant (0.08mls), Alum (Pierce and Warriner, Chester, UK). The protein antigen was firstly diluted in PBS and to this solution Alum was added in a dropwise manner. The antigen/adjuvant solution was then mixed over a 30 minute period.

4 weeks following primary immunisation mice were bled. 10 drops of blood were collected from the retro-orbital sinus into 1.5ml Eppendorff tubes via heparinised capillary tubes (Hawksley and sons Ltd, Sussex, UK). Eppendorff tubes containing blood were left at room temperature for 1 hour before being incubated overnight at 4^{o}C to allow the blood clot to form and separate from the sera. After removal of the blood clot, with forceps, any remaining RBCs were pelleted by centrifugation and sera collected into fresh tubes. Sera were stored at -70^{o}C until use.

Mice were re-immunised 7 and 9 weeks after the primary immunisation. 7 days after each of these immunisations mice were bled and sera collected as described above.

The injection and bleeding protocol is summarised in Fig. 2.12.

Fig. 2.13. Comparison of ELISAs using developing reagents from different sources.



—▲— "sigma" urease conjugate with Rabbit 669 anti-BLG serum
—○— "sera-lab" urease conjugate with Rabbit 669 anti-BLG serum
—■— "sigma" urease conjugate with normal Rabbit 669 serum
—□— "sera-lab" urease conjugate with normal Rabbit 669 serum

For both "sigma" and "sera-lab" reagents non-specific binding was only seen at low dilutions (both had an endpoint of 2) and equivalent endpoint dilutions for specific binding (9).

b) Analysis

The antibody titre of each serum sample was estimated using the ELISA technique previously described. In brief, serum samples from immunised mice were diluted 1/10 in DBF and then double diluted to a dilution of 1/20480. The diluted sera were then added to the appropriate wells of a previously coated, and blocked, ELISA plate. Each sample was tested in triplicate. The second step antibody was an anti-mouse IgG (whole molecule) and was used at a dilution of either 1/100 (SERA-LAB) or 1/1000 (SIGMA). Finally the substrate was added and the plates incubated for 30 (SERA-LAB) or 45 minutes (SIGMA) at 37°C. The second step antibody concentration and substrate development time varied with reagents purchased from different companies. However both gave similar OD values for most dilutions of Rabbit 669 anti-ovine BLG sera as well as the same endpoint titre see Fig. 2.13.

It should be noted that in the following results sections that no pre-bleed data is shown. All pre-bleed sera analysed had no detectable anti-ovine BLG, anti-bovine BLG or anti-OVA reactivity.

ii) T Cells

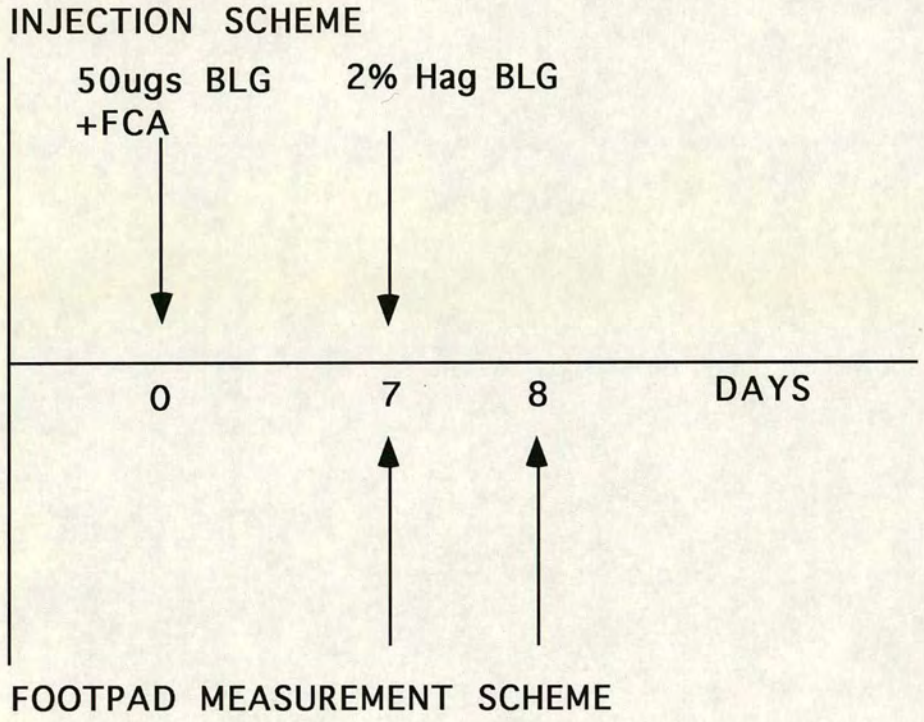
a) Immunisation

Following halothane anaesthesia, each mouse was injected, in the left hind footpad using a 27G needle (Becton Dickinson UK Ltd, Oxford), with a total volume of 50μls containing 50μgs of antigen plus adjuvant; 50μgs of antigen was diluted in 25μls of PBS and emulsified with 12.5μls of FCA using an omnimixer (Sorvall, Newtown, USA). 12.5μls of 2% Tween 80 was then added and the solution remixed.

b) In vivo T cell responses (see Fig. 2.14)

7 days after the primary footpad injection mice in experimental groups received a challenge dose of antigen, 50μls of 2% heat aggregated protein solution, in the right hind footpad using a (21G). Control mice were given a PBS challenge. A 2% protein solution was made up in PBS and this

Fig. 2.14. Injection and footpad measurements for T cell responses



Mice were injected (sc) as described above.

solution was added to a 70°C water bath for one hour to aggregate the protein. The solution was then cooled at room temperature before being used.
Immediately prior to challenge the right footpad was measured using callipers (Draper, Japan) and the footpad thickness noted. 24 hours after challenge the footpad was remeasured and the response calculated by subtracting the footpad measurement before injection with the measurement following challenge for each mouse in the experimental and control groups. The mean footpad difference +/- standard deviations was calculated for each group.

c) In vitro T cell responses
7 days after primary immunisation mice were killed and the popliteal lymph node draining the immunised footpad was removed sterilely. For each node a cell suspension of 2.5 x 10^6 cells per ml was made in sterile RPMI-FCS (Gibco, Paisley, UK) and 100μls of this suspension dispensed into 15 wells of a sterile 96 well culture plate (Corning, Bibby Sterilin Ltd, Staffordshire, UK). A range of antigen concentrations were made in sterile RPMI-FCS; 1000, 500 and 100μgs/ml. 100μls of each antigen concentration was then added to the appropriate 3 wells containing cells. 100μls of media as well as 4μgs of Con A were added to 3 control wells. Plates were then incubated for 3 days at 37°C/CO_2 before 1μCi of ^{3}H-Tritium (Amersham International Plc, Buckinghamshire, UK) was added per well. Plates were reincubated for a further 24 hours before the cells were harvested, using a cell harvester (Hewlett Packard, USA), onto fibre-glass filters (ICN Biomedicals Inc, California, USA) which were dried at 37°C before being placed into scintillation fluid (National Diagnostics, New Jersey, USA). The amount of ^{3}H incorporated per well was counted in a beta counter (Hewlett Packard, USA) and the counts per minute (CPM) noted for each sample.

iii) Bovine BLG oral immunisation

8-12 week old CBA/Ca male and female mice were orally challenged with bovine BLG dissolved in sterile dH_2O supplied as drinking water. Dosage and exposure time varied. Some mice received 25mgs of bovine BLG for 24 hours or 21 days whilst others received 50mgs of bovine BLG for 24 hours or 21 days. Three weeks after the end of the feeding regimen mice were immunised with 50μgs of bovine BLG plus FCA in the left hind footpad (see above). 2 weeks later mice were bled for sera, as described, and challenged with 2% heat aggregated bovine BLG in the right hind footpad. Immediately prior to and 24 hours after challenging footpad measurements were taken (see in vivo T cell immunisation procedures). 7 days following the secondary challenge mice were bled for sera. Antibody titres were analysed using bovine BLG coated ELISA assays.

2.5) Bone marrow transplantation

i) Radiation dosage.

Three groups of 4 non-transgenic mice were exposed to 8, 9 or 10.5Gy of gamma irradiation from a ^{137}Cs source (Nuncatom, London, UK). Exposure to 10.5Gy resulted in death 9-12 days later and this dose was chosen for future experiments.

ii) Bone marrow preparation.

Bone marrow donor animals were killed by ether anaesthesia or cervical dislocation. Femora, humeri and tibiae bones were dissected out and excess muscle removed. The epiphyses of each bone was cut off with fine scissors and bone marrow flushed out by passing 1ml of sterile PBS through the centre of each bone via a 25G (Becton Dickinson UK Ltd, Oxford) needle. A single cell suspension was produced by aspiration and explusion of bone marrow flushings through decreasing needle gauges, from a 19G to a 25G needle. The cell count was determined using a Coulter counter (Coulter Electronics Ltd, Luton, UK) and adjusted to give a final concentration of 1 x 10^7 cells per 0.4ml of sterile PBS.

iii) Mouse immunisations.

Following irradiation, recipient mice received $1x10^7$ donor cells intravenously into the lateral tail vein using a 25G needle. Animals were kept on neomycin sulphate treated water for 7 days following irradiation. Control mice, not receiving bone marrow, died 9-12 days after irradiation.
3 months following bone marrow transfer mice were immunised for antibody as described (see section 2.3).

2.6) STATISTICS

i) Antibody responses.
Antibody titres were analysed using a non-parametric test, the Mann Whitney test, on the Minitab software. Significance was taken as $p<0.05$ at a 95% confidence level.

ii) T cell responses
T cell responses following footpad immunisation and challenge were analysed using a 2-sample T-test on the Minitab software. Significance was taken as $p<0.05$ at a 95% confidence interval.

CHAPTER 3

THE EFFECT OF MHC HAPLOTYPE ON IMMUNE RESPONSES TO BLG

Early experiments using guinea pigs immunised with a synthetic homopolmer PLL coupled to DNP demonstrated that these animals either made good antibody and T cell responses to this molecule (responder animals) or they did not respond at all (non-responders) [319-321]. Differences in response to synthetic polymers were also shown in mice [322-325]. Mice of haplotype H-2^p, H-2^q and H-2^s failed to respond, at both the B and T cell level, to polyGlu^{60} Ala^{30} Tyr^{10} (GAT) whilst mice with the haplotype H-2^a, H-2^b and H-2^d did respond to this molecule. Analyses of the immune responses to another synthetic polymer, polyGlu^{50}Tyr^{50} (GT) showed that over 40 inbred strains of mice were unresponsive to this molecule [326]. However it was found that bm12 mice respond to GT by producing anti-GT antibodies [327].

Immune response studies have also been extended to many other protein molecules which are presented by either Class 1 or Class 2 MHC molecules. Mice possessing certain alleles of Class 1 and Class 2 MHC do not respond to particular antigens: 1). K^b and D^d expressing mice do not respond to the H-Y male antigen. 2). D^d, K^b, D^k and L^d expressing mice are non-responsive to the influenza nucleoprotein. 3). I-A^k expressing mice are unresponsive to bovine insulin. 4). I-A^d mice do not respond to HEL and 5). I-E^d mice do not respond to pigeon cytochrome c.

The reverse is also true. Mice possessing certain MHC alleles are responsive to certain proteins: 1). D^b and K^k expressing mice respond to the H-Y antigen. 2). Mice possessing D^b or K^d respond to the influenza nucleoprotein. 3). I-A^b mice respond to bovine insulin. 4). I-A^k respond to HEL and 5). I-E^k mice respond to pigeon cytochrome c [328].

Genes determining responsiveness and non-responsiveness were named immune response genes.
Recently it has been shown that both Class 1 and Class 2 MHC molecules could bind to a diverse range of peptides but not every peptide, for example I-A^k can bind the immunodominant peptide of HEL whilst I-A^d does not bind this molecule. Binding of the peptide to correct MHC is a crucial step for T cell recognition and ultimately the ability to induce both antibody and T cell responses. Non-responsiveness in some cases appears to be a failure in the binding or presentation of peptides although in others presentation can occur but no T cells capable of recognising this complex are present [328].
Since the original transgenic stocks were on a mixed MHC background, systemic antibody and T cell responses to purified ovine BLG were investigated in mice possessing relevant MHC haplotypes. The influence of sex on the immune response was also analysed. Ovalbumin was used as a positive control antigen.

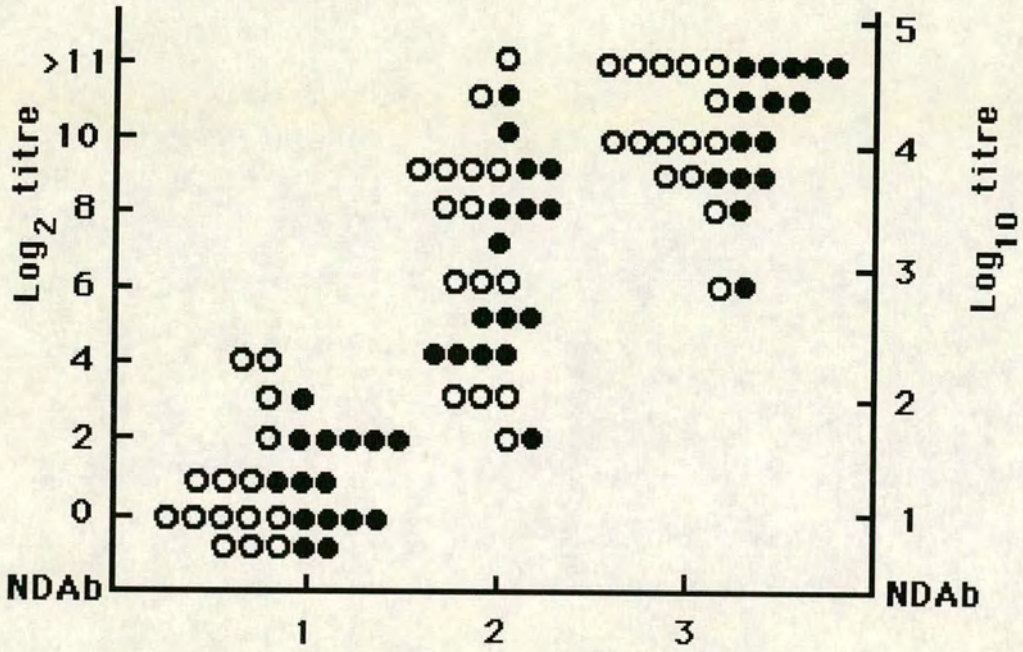
3.1) Antibody responses to ovine BLG

Female and male mice expressing different MHC genes were investigated for their ability to mount antibody responses to ovine BLG. CBA/Ca (H-2^k), C57BL/6 (H-2^b), BALB/c (H-2^d) and C57BL/6 X CBA/Ca (H-2^{kxb}) were immunised intraperitoneally (ip) for antibody production as described (see Chapter 2).
Mice were bled at various time points following primary and secondary challenges (see Chapter 2) and IgG antibody levels analysed using a BLG-specific ELISA. The secondary step antibody used in this assay was an anti-IgG whole molecule. Theoretically this antibody would cross-react with the light chains of any IgM present in the serum samples following primary immunisation since the dominant antibody class produced would be IgM. This was overcome by analysing the primary response 28 days following immunisation. The specificity of the secondary antibody should not interfere with the antibody results following challenge since the predominant antibody in a secondary response is IgG.

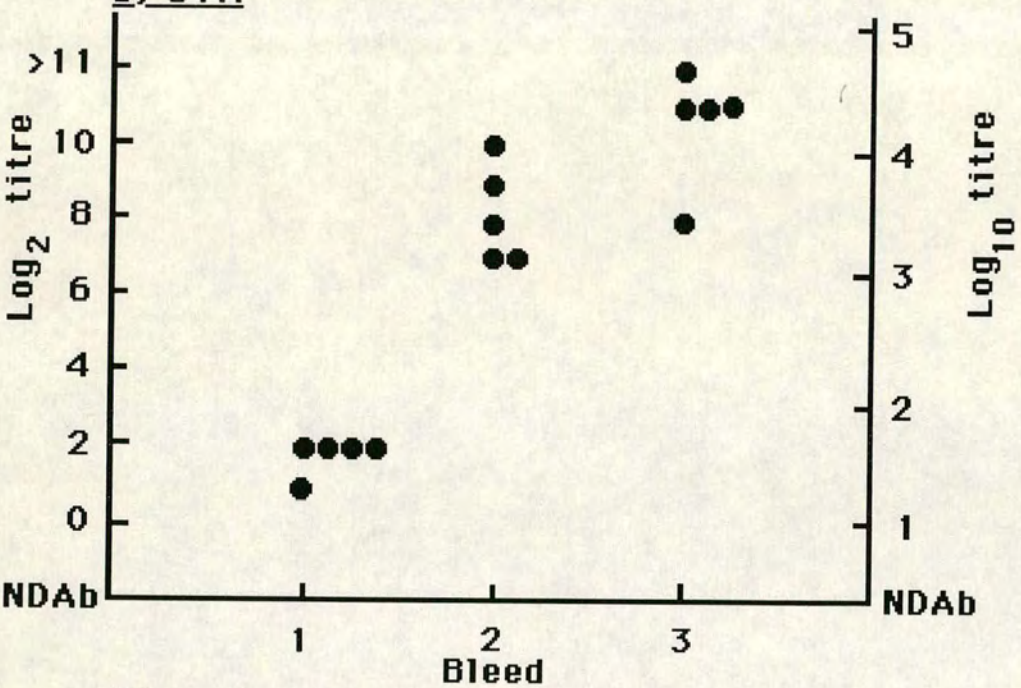
Fig. 3.1.

Antibody responses of CBA/Ca males (○) and females (●) to ovine BLG and OVA. Each point is the data for serum from a single mouse

a) ovine BLG



b) OVA

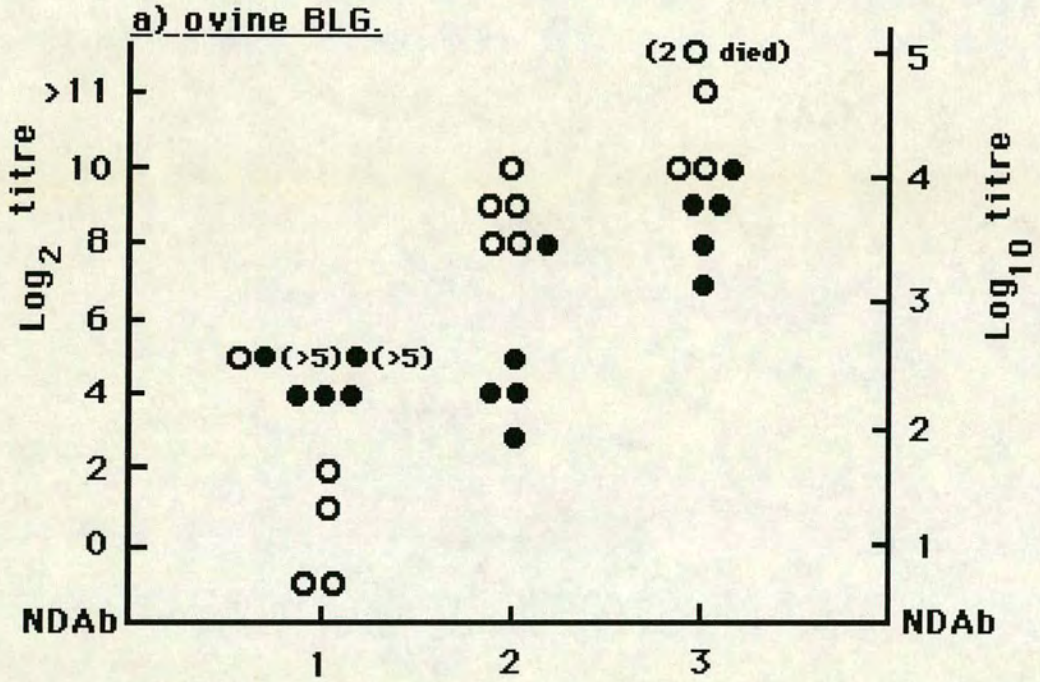


NDAb= no detectable antibody

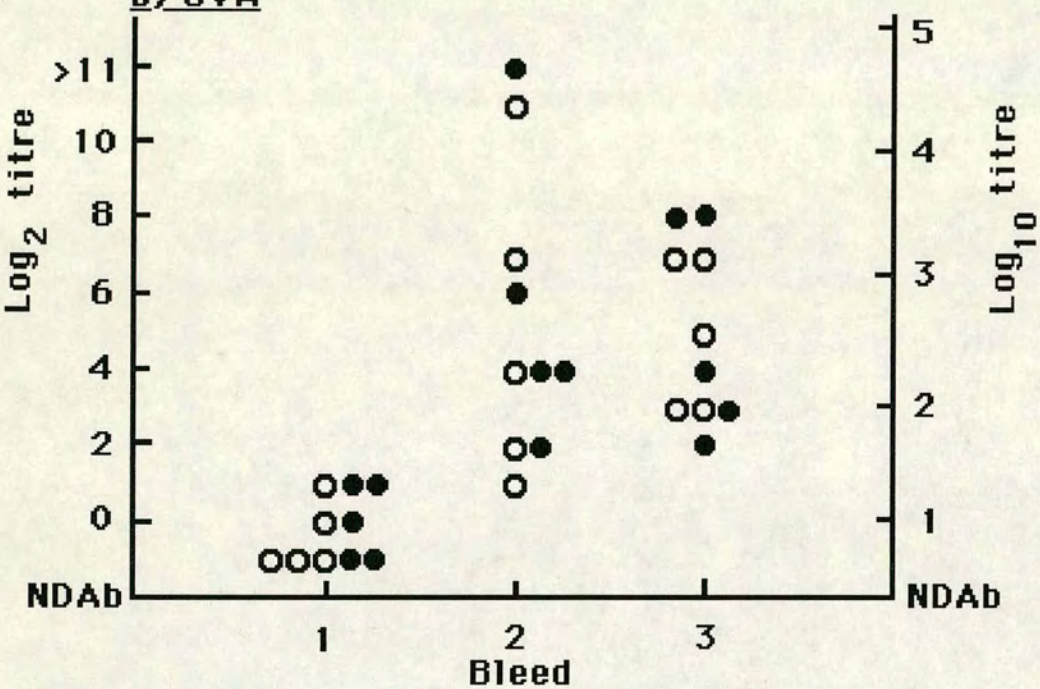
Fig. 3.2.

Antibody responses of C56BL/6 male (O) and female (●) mice to ovine BLG and OVA. Each point is the data for serum from a single mouse.

a) ovine BLG.



b) OVA



NDAb= no detectable antibody.

Figures 3.1a, 3.2a, 3.3a and 3.4a illustrate the anti-ovine BLG IgG titre detected in the sera of ovine BLG immunised mice, each mouse being represented as a single point, for CBA/Ca, C57BL/6, CBA/Ca x C57BL/6 F1 and BALB/c respectively. Primary, secondary and tertiary antibody responses are shown.

i) CBA/Ca mice (Fig. 3.1a).

Figure 3.1a represents the anti-BLG responses of CBA/Ca male and female mice from 2 experiments. The analysis is from the pooled data.

Male and female CBA/Ca mice were immunised with ovine BLG and the primary and secondary anti-BLG IgG titres were analysed by ELISA. Female and male CBA/Ca mice made a small, but significant amount of anti-BLG IgG antibody following primary immunisation with this antigen in adjuvant compared to mice challenged with ovine BLG and saline (data not included).

Following a second immunisation the anti-BLG IgG response increased significantly ($p<0.01$) compared to the primary response, for both male and female CBA/Ca mice. Significant increases in the response to ovine BLG were also found after a third immunisation in male CBA/Ca mice ($p<0.01$) and female CBA/Ca mice ($p<0.001$). However at an individual experimental level a significant increase only occurred in one experiment.

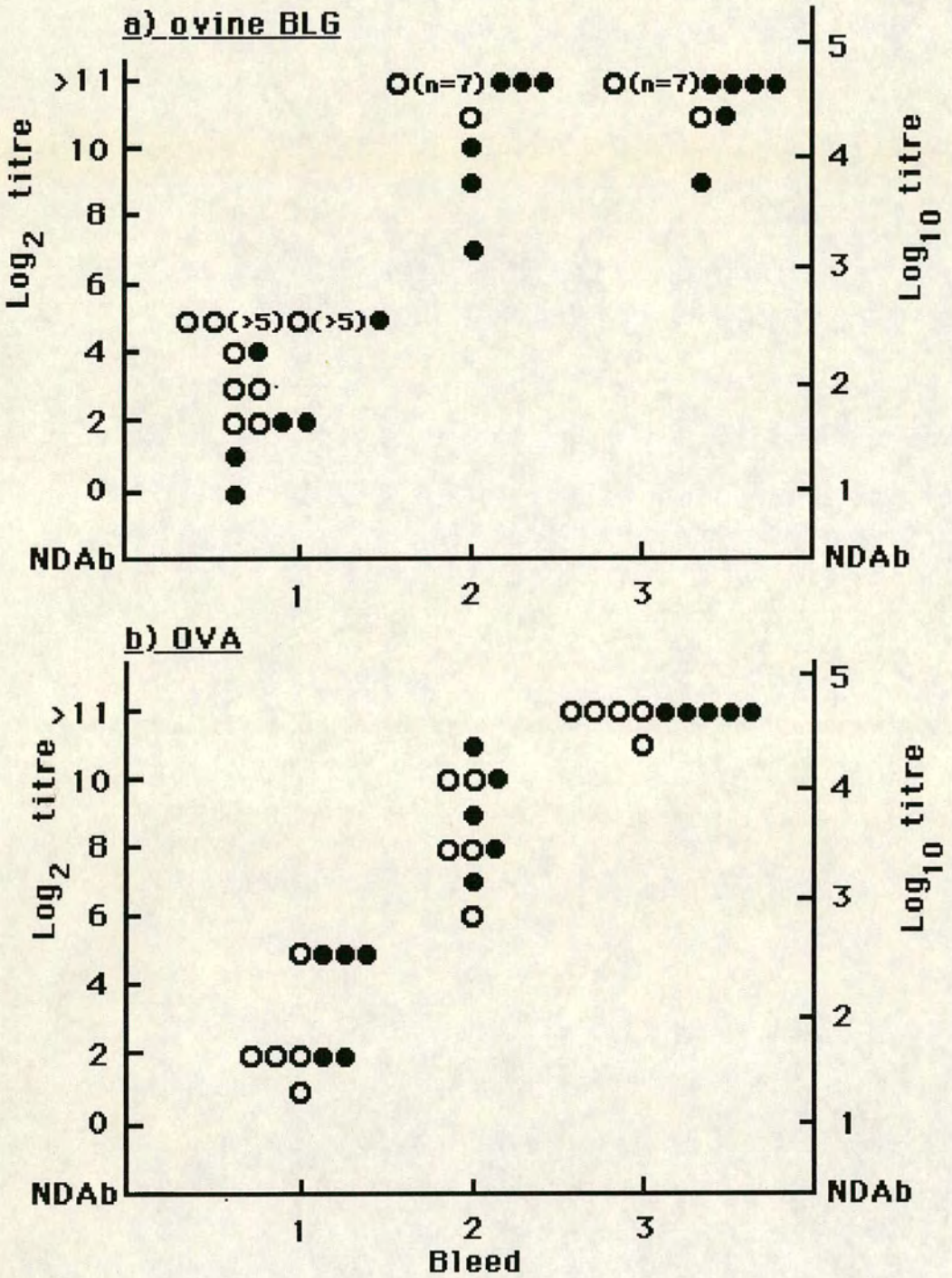
In conclusion, CBA/Ca mice responded to both primary and secondary immunisation with ovine BLG plus Alum by producing anti-ovine BLG specific IgG antibodies. Titres of these antibodies were low following primary immunisation however they increased following a second and third BLG immunisation. This increase in IgG response is characteristic of a memory response. In both experiments male and female primary and secondary titres were comparable.

ii) C57BL/6 mice (Fig. 3.2a)

The data shown in Fig.3.2a is from one experiment. Following a secondary immunisation with BLG there was a significant increase in the response for male ($p<0.01$) C57BL/6 mice. This

Fig. 3.3

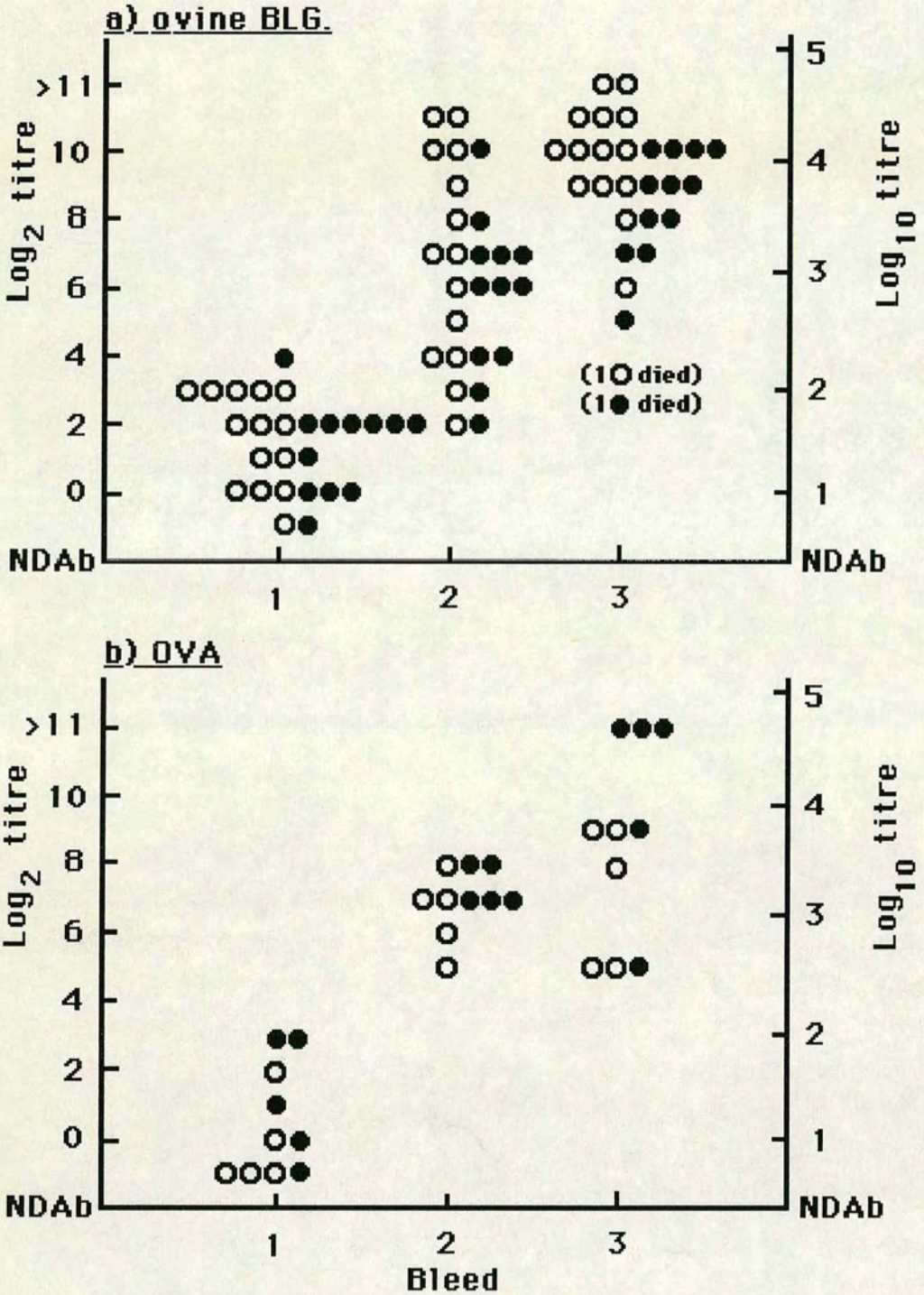
Antibody responses of F1 male (O) and female (●) mice to ovine BLG and OVA. Each point is the data for serum from a single mouse



NDAb= no detectable antibody

Fig 3.4

Antibody responses of BALB/c male (O) and female (●) mice to ovine BLG and OVA. Each point is the data for serum from a single mouse.



NDAb= no detectable antibody

was not found for female mice however following a third immunisation a significant increase in anti-BLG responses did occur ($p<0.05$). No further increase in the antibody response occurred following a third immunisation for male C57BL/6 mice.

Male and female C57BL/6 mice following a first and third immunisation made equivalent antibody responses. However after a second immunisation male mice made a significantly greater antibody response than female C57BL/6 mice ($p<0.05$).

iii) CBA/Ca x C57BL/6F1 mice (Fig. 3.3a).

Figure 3.3a shows the anti-ovine BLG responses of F1 mice from one experiment. Following a primary immunisation the anti-BLG titres ranged from 0 to 5 for female and 2 to >5 for male F1 mice. A significant increase in BLG-specific antibody titres occurred following a second immunisation for both male (titre range 11 to >11, $p<0.001$) and female (titre range 7 to >11, $p<0.01$) F1 mice. However no significant increases in anti-BLG titres occurred following a third challenge for either male or female mice.

In conclusion, F1 male and female mice can respond to ovine BLG following a primary or secondary immunisation by producing high titres of anti-BLG specific antibody and both female and male F1 mice made comparable antibody responses to this protein.

iv) BALB/c mice (Fig. 3.4a)

The anti-BLG responses shown in Fig. 3.4a are data from two experiments. The observations and p values described below are for the pooled data.

Following a primary immunisation with ovine BLG male and female BALB/c mice producing low anti-BLG titres (titres ranging from no detectable antibody to 4 for females and from no-detectable antibody to 3 for males). A significant increase in anti-BLG IgG titres occurred following a second BLG immunisation for both male ($p<0.001$) and female ($p<0.0001$) BALB/c mice.

Although at an individual experiment level no significant increase in antibody levels occurred following a third immunisation in one experiment pooling the data revealed a significant increase in antibody titres for male ($p<0.05$) and female ($p<0.01$) mice following a third immunisation.
BALB/c males and females made comparable anti-BLG IgG responses after each immunisation.

3.2) Antibody responses to Ovalbumin.

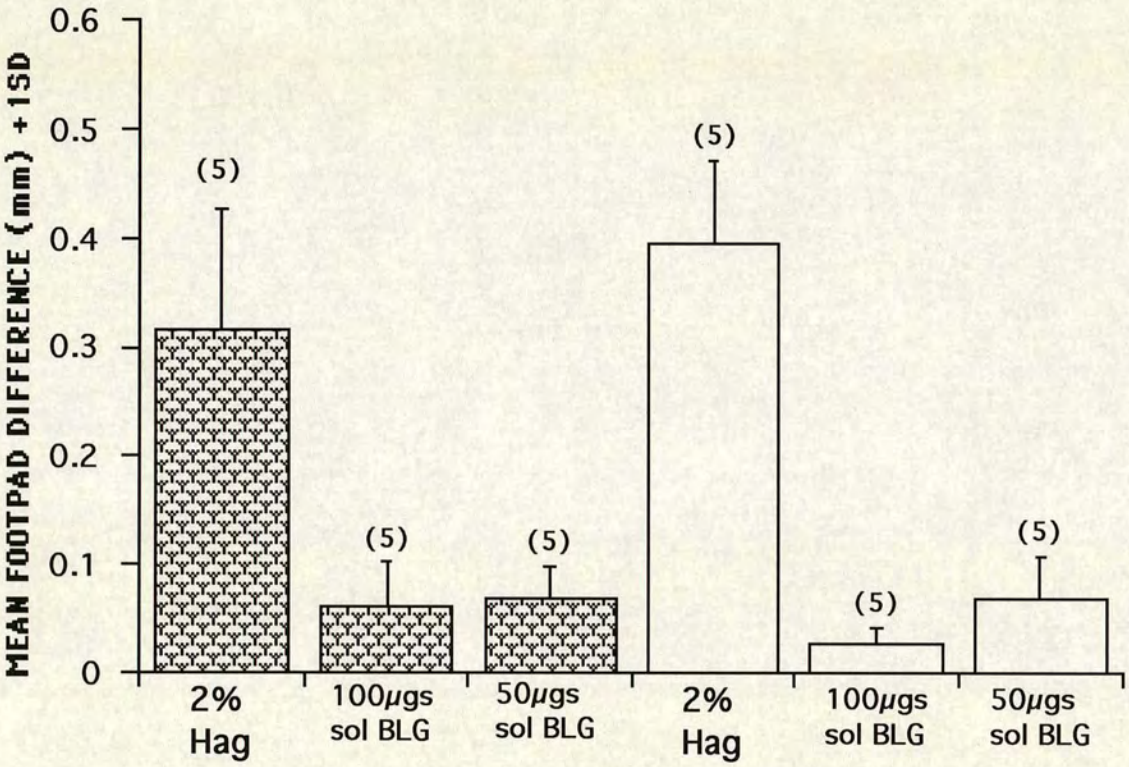
Groups of mice were immunised with a "control" antigen Ovalbumin (OVA), plus Alum. The primary and secondary response to OVA for CBA/Ca, C57BL/6, F1 and BALB/c male and female mice are shown in Figures 3.1b, 3.2b, 3.3b and 3.4b respectively. Each mouse is represented as one point and the data from one experiment.
CBA/Ca females (no males were tested) and both sexes from C57BL/6, F1 and BALB/c mice made low anti-OVA IgG responses following primary immunisation. Following a secondary immunisation a significant increase in anti-OVA specific IgG ($p<0.01$ for CBA/Ca females, $p< 0.05$ for C57BL/6 male and female mice, $p<0.01$ for F1 male and female mice and $p<0.05$ and $p<0.01$ for BALB/c males and females respectively) occurred. After a third challenge with OVA only the F1 mice and female CBA/Ca mice significantly increased their anti-OVA titres ($p<0.01$ for male and female F1 and $p<0.05$ for CBA/Ca females). This was not found for BALB/c or C57BL/6 mice.
Thus male and female F1, C57BL/6 and BALB/c and female CBA/Ca mice can produce equivalent primary and secondary anti-OVA IgG responses following immunisation with OVA plus Alum adjuvant. Male and female mice making comparable responses.

3.3) Summary of antibody data

All groups of mice tested made good primary and secondary responses to ovine BLG when injected ip with the adjuvant Alum. In both cases there was variation in antibody titre

Fig. 3.5

DTH responses to heat aggregated (Hag) vs soluble bovine BLG.



Mice were immunised with 50μgs or 100μgs of bovine BLG + FCA and 7 days later were challenged with either soluble (50 or 100μgs/ml) or 2% Hag bovine BLG.

(nos)= number of mice tested

Error bars are + 1SD

50μgs of bovine BLG

100μgs of bovine BLG

levels for individual mice in each group. This could not be attributed to the sex of the responding animal, since male and female mice of each group made comparable IgG responses to ovine BLG challenges. The data indicated that this protein was immunogenic in all strains of mice tested.

3.4) T cell responses to bovine BLG. (Individual T cell responses are shown in Appendix 8).

The secondary responses of T cells to BLG were analysed in vivo using a simple and rapid protocol, i.e. measurement of a DTH response via footpad thickening.

In order to preserve our stocks of purified ovine BLG, initial DTH experiments were performed using bovine BLG which is commercially available and at the antibody level at least immunologically cross-reactive with ovine BLG (see Chapter 4).

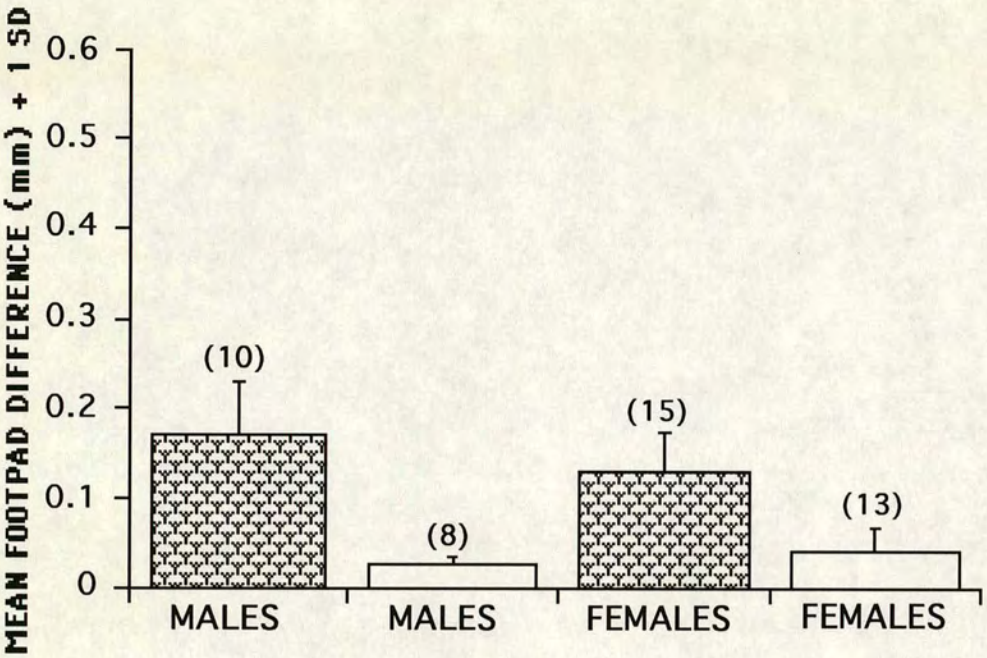
CBA/Ca female mice were primed with either 100μgs or 50μgs of bovine BLG plus FCA in their left hind footpad and seven days later they were challenged with either soluble or heat aggregated bovine BLG in the right footpad. 24 hours post challenge the thickness of the right footpad was measured using callipers. The difference in footpad thickening pre and post challenge corresponded to the secondary T cell response to the BLG protein. The mean footpad differences, in millimeters, and standard deviation (SD) of the means for each experimental group were calculated and are shown in Fig. 3.5.

Challenge with a soluble antigen, regardless of initial priming dose, gave poor secondary responses. However good secondary T cell responses were evident following a challenge with a heat aggregated antigen and again this was not significantly influenced by the priming dose.

Systemic T cells, from female CBA/Ca mice, can therefore be primed and respond to a secondary challenge with bovine BLG. This response was dependent on the antigen being aggregated for secondary immunisation, presumably since large protein aggregates are retained in the skin [329]. Both 100μg and 50μg priming doses resulted in comparable T cell responses

Fig. 3.6a

T cell responses of CBA/Ca mice to ovine BLG

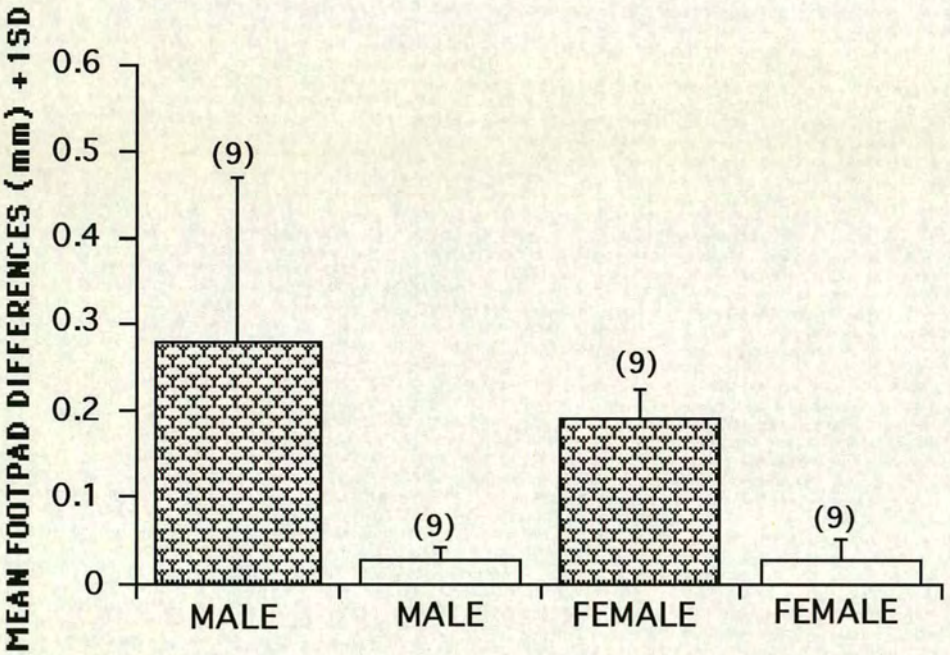


Mice were immunised with ovine BLG+FCA and 7 days later challenged with 2% Hag ovine BLG or PBS.
(nos) = number of mice tested.
Error bars are +1SD

▨ 2% Hag ovine BLG
□ PBS

Fig. 3.6b

T cell responses to ovine BLG for C57BL/6 mice



Mice were immunised with ovine BLG+FCA and 7 days later were challenged with either 2% Hag ovine BLG or PBS.
(nos) = number of mice tested.
Error bars are +1SD

▨ 2% Hag ovine BLG
□ PBS

following challenge, and for this reason 50μgs of antigen was designated the priming dose for future T cell experiments.

3.5) T cell responses to ovine BLG.

Female and male CBA/Ca, C57BL/6, CBA/Ca X C57BL/6 F1 and BALB/c mice were immunised as described above with ovine BLG. Control mice were challenged with PBS whilst experimental groups were challenged with 2% heat aggregated ovine BLG. The mean+1SD of pooled data from two experiments (unless otherwise stated) are shown in Figs 3.6a, b, c and d. The mean footpad differences of the experimental groups were compared with the equivalent values of PBS controls using a 2-sample T-test available on Minitab software.
In the following sections the observed results and p values are from the data of 2 pooled experiments. The observations made were also noted at the level of the individual experiment unless indicated. Individual T cell data are shown in Appendix 9.

i) CBA/Ca mice (Fig. 3.6a)

Compared to their respective controls, male and female CBA/Ca mice, made small but significant secondary T cell responses to ovine BLG ($p<0.001$). Male and female CBA/Ca made equivalent T cell responses to this protein.

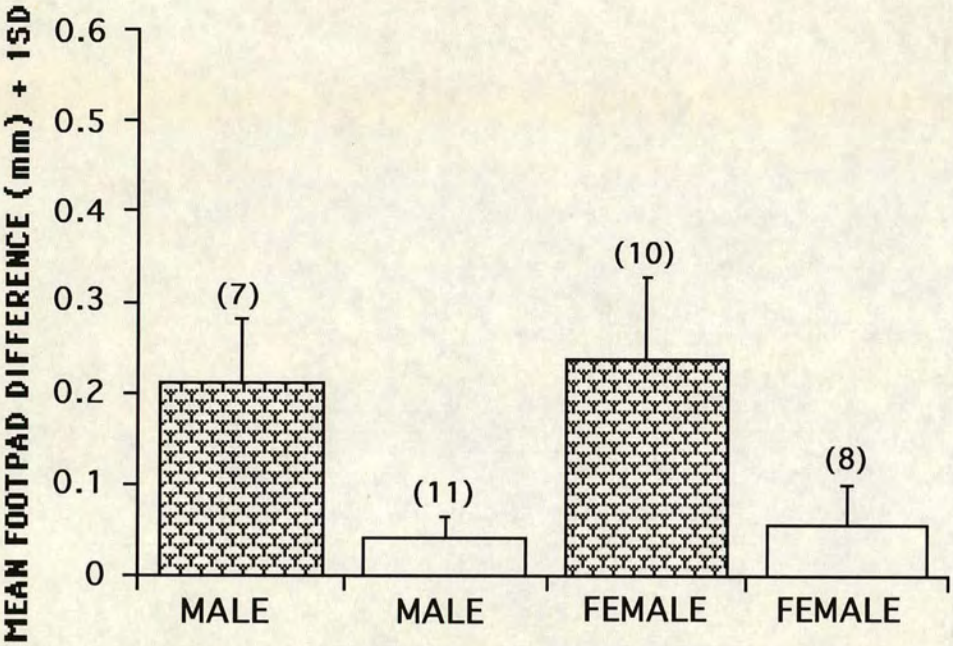
ii) C57BL/6 mice (Fig. 3.6b).

Male and female C57BL/6 mice made significant T cell responses to ovine BLG ($p<0.01$ and $p<0.001$ respectively) as compared to control mice. Thus suggesting that C57BL/6 mice can produce a T cell response to ovine BLG following this immunisation protocol. Males in experiment one made a greater response than females ($p<0.05$) whilst the opposite was observed in the second experiment. However when the data from both experiments was pooled no significant difference in responsiveness was observed between the sexes.

iii) F1 mice (Fig. 3.6c).

Fig. 3.6c

T cell responses to ovine BLG for F1 mice

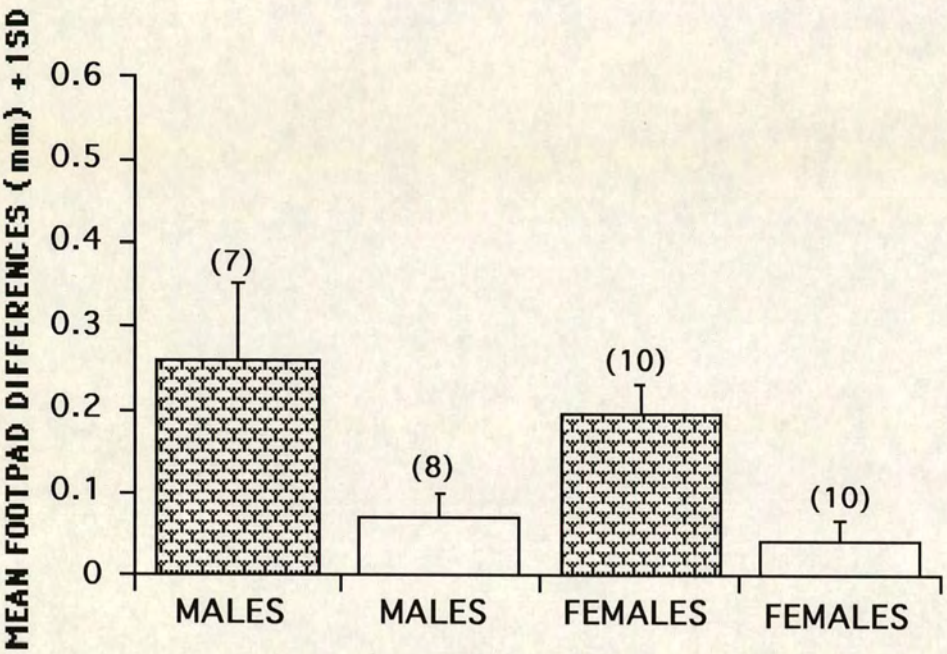


Mice were immunised with ovine BLG+FCA and 7 days later challenged with 2% Hag ovine BLG or PBS.
(nos) = number of mice tested.
Error bars are +1SD

▨ 2% Hag ovine BLG
□ PBS

Fig. 3.6d

T cell responses of BALB/c mice to ovine BLG



Mice were immunised with ovine BLG+FCA and 7 days later challenged with 2% Hag ovine BLG or PBS.
(nos) = number of mice tested.
Error bars are +1SD

▨ 2% Hag ovine BLG
□ PBS

The footpad measurement for F1 males in Fig. 3.6c is from one experiment only. Comparing the experimental mean footpad difference with control data indicated that male F1 mice were capable of producing significant T cell response to ovine BLG ($p<0.001$).
The T cell data in Fig.3.6c for female F1 mice is pooled from two experiments. Comparing the experimental female data with that of control data indicated that female F1 mice made a significant T cell response to this protein ($p<0.001$). Comparing the T cell responses of F1 male and female mice indicated that both sexes made equivalent T cell responses to ovine BLG.

iv) BALB/c mice (Fig. 3.6d).
Figure 3.6d represents the T cell responses of male and female BALB/c mice from two experiments. Both male and female BALB/c made significant ($p<0.001$) T cell responses to ovine BLG as compared to control mice. Male and female BALB/c mice made equivalent T cell responses to this protein.

3.6) Summary of T cell data

Male and female mice, regardless of MHC background respond to ovine BLG and produce small but significant *in vivo* T cell responses to this protein as measured by a DTH reaction.

CHAPTER 4.

BLG-SPECIFIC ANTIBODY RESPONSES IN BLG-TRANSGENIC MICE

Virgin BLG-transgenic female and male mice as well as exbreeder BLG-transgenic females variously exposed or not to BLG in milk or whey protein in their diet were immunised parenterally with ovine BLG and the antibody responses measured by an ELISA techique described in Chapter 2.

4.1) F1 BLG-transgenic offspring from a homozygous transgenic male x CBA/Ca female.

The first group of transgenic mice tested for antibody responsiveness to ovine BLG were derived from mating line 45 homozygous BLG-transgenic males with CBA/Ca females. All offspring from this mating, the F1 BLG-transgenics, carried the ovine BLG gene as shown by PCR and BLG specific primers (see Chapter 2). To test for BLG gene expression RNA extraction was performed on various tissues from BLG-transgenic mice. Following gel electrophoresis and Northern blotting the presence of BLG RNA was analysed by using a BLG cDNA probe [293]. Expression of BLG RNA was only evident in lactating mammary tissue from BLG expressing female mice, as previously described [293] see Figs 4.1a and 4.1b. No BLG RNA expression was evident in either the thymus or lymph nodes of BLG-transgenic mice, see Figs 4.1a and 4.1b.

At three months of age, these mice were immunised with either ovine BLG, bovine BLG, or OVA, as described and any resulting serum IgG titres were analysed using an ELISA. Plates were coated with either ovine or bovine BLG or OVA, respectively. Each mouse serum was tested in triplicate and the antibody titre plotted. The anti-BLG titres derived from the sera of these BLG-transgenic mice were compared with the anti-BLG response of CBA/Ca mice, these mice having been

Fig. 4.1a RNA gel and Northern Blot

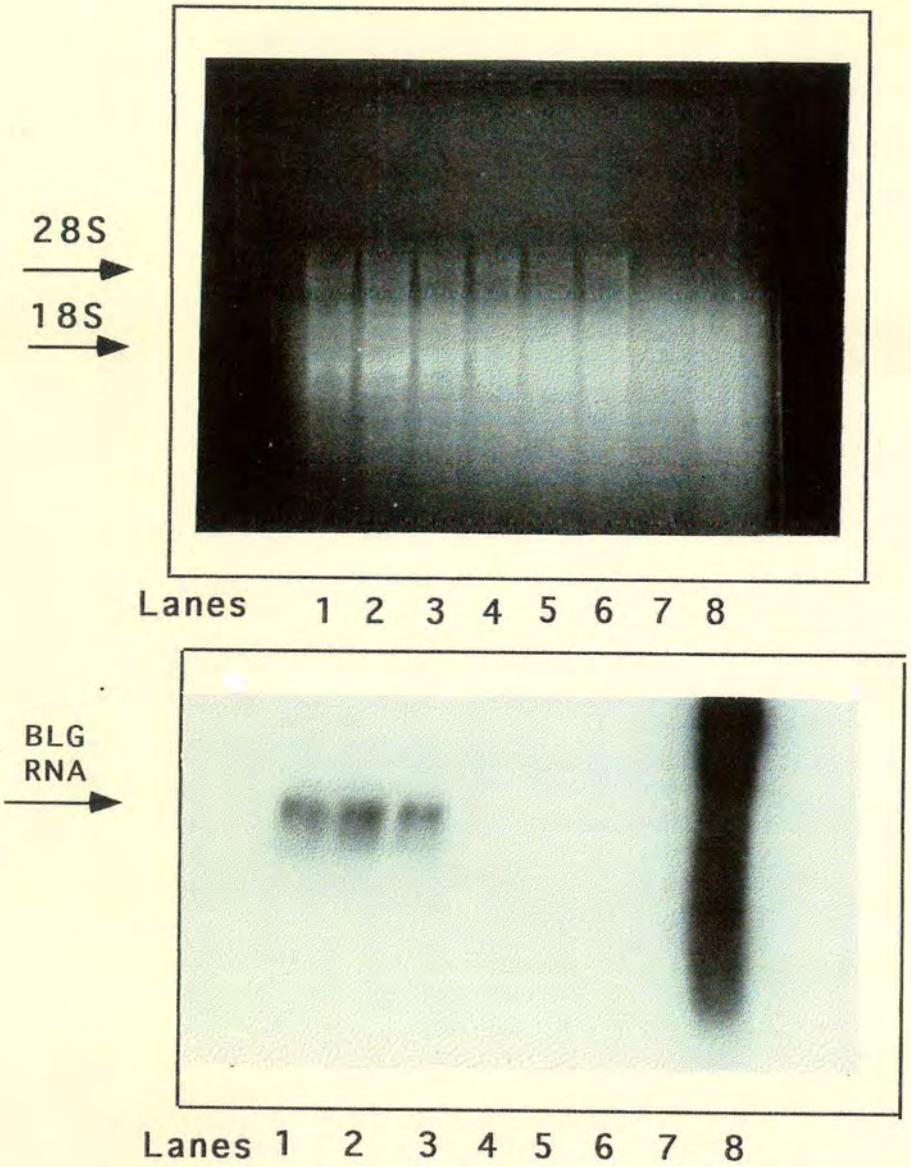


Fig. 4.1a represents pictures of 1) an ethidium bromide and UV exposed formaldehyde gel containing RNA samples (upper picture) and 2) a Northern blot of the gel following labelling with a BLG cDNA probe (lower picture).

Lanes 1, 2 and 3 contains RNA from the mammary glands of three lactating transgenic females. Lane 4 contains thymic RNA from a lactating BLG-transgenic female, lanes 5 and 6 contains thymic RNA from two CBA/Ca males. Lane 7 contains no RNA. An RNA ladder (SIGMA) was added to lane 8. It was noted that the BLG cDNA probe bound to the RNA ladder.
5μgs of RNA added per well.

Fig. 4.1b RNA Gel and Northern Blot

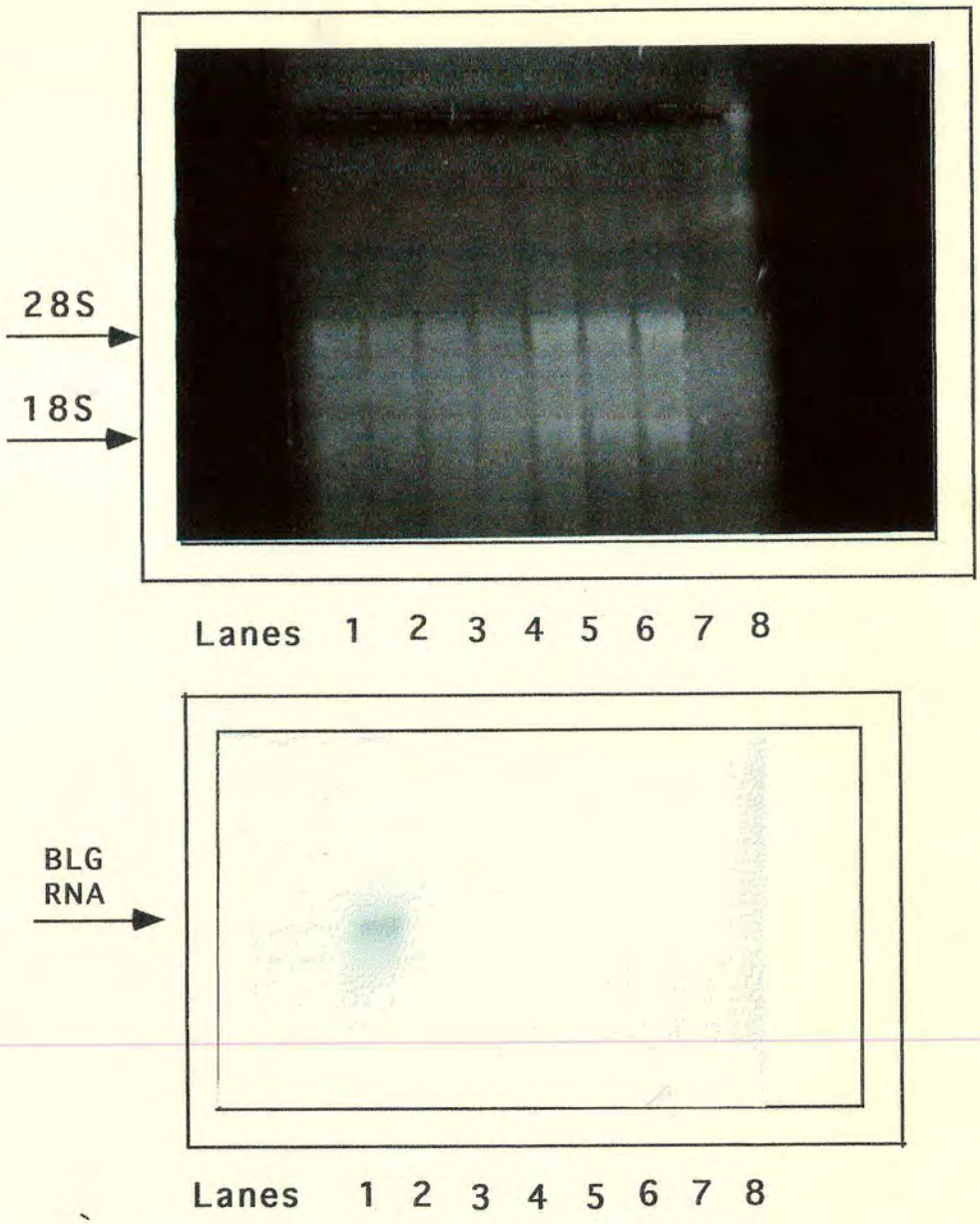
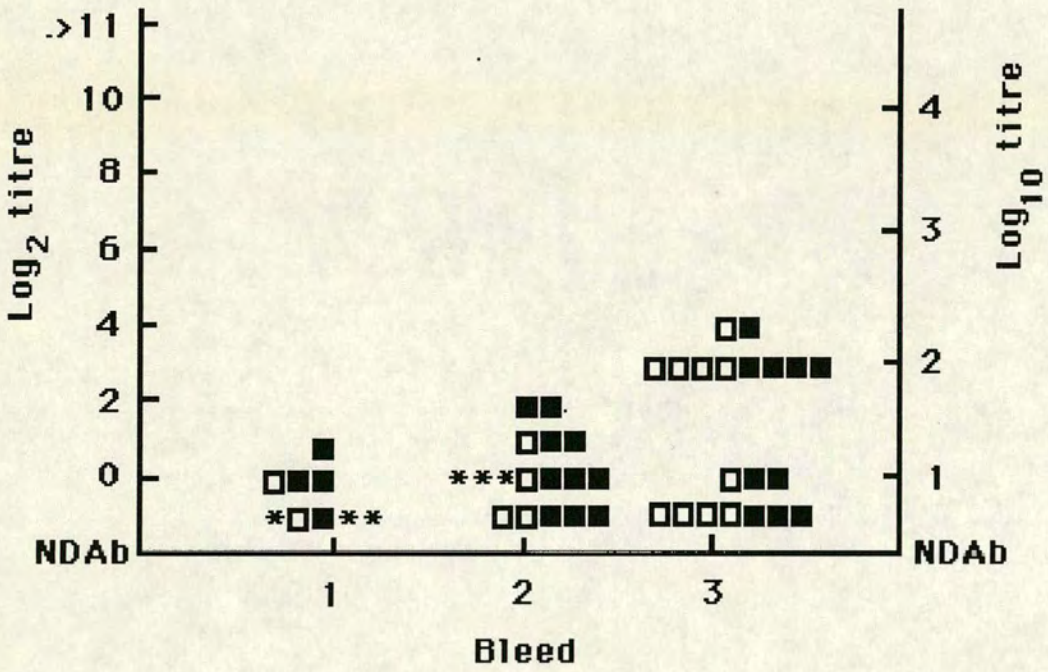


Fig. 4.1b represents pictures of 1) an ethidium bromide and UV exposed formaldehyde gel containing RNA samples (upper picture) and 2) a Northern blot of this gel following labelling with a BLG cDNA probe (lower picture).
Lane 1 contains RNA from the mammary gland of a lactating BLG-transgenic female. Lanes 2 and 4 contain RNA from BLG-transgenic male and females lymph nodes, respectively. Lanes 3 and **5** contain thymic RNA from virgin female and male BLG-transgenic mice respectively.
Lanes 6 and 7 contain RNA from BLG-transgenic fetal thymus and liver respectively. Lane 8 is the RNA ladder. Lane 8 was removed from the Northern blot picture however the probe bound as shown in Fig 4.1a.
5μgs of RNA added per well.

Fig 4.2a

Antibody responses of F1 BLG-transgenic male (□) and female (■) mice to ovine BLG. Each point is the data for serum from a single mouse.

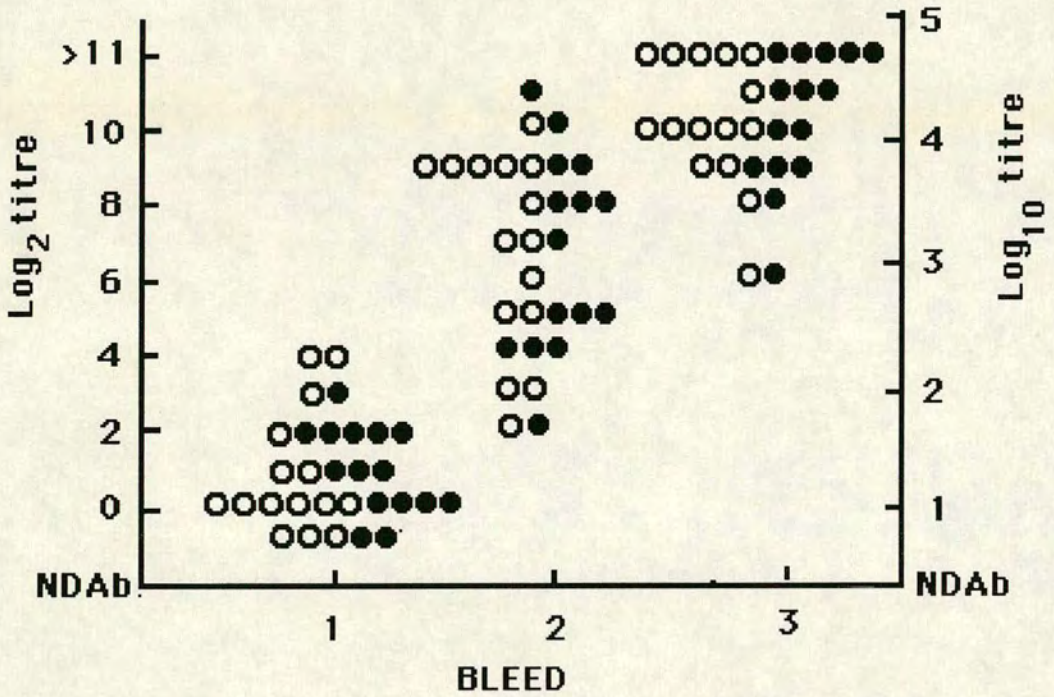


*□n=9
**■n=7
***□n=7

NDAb= no detectable antibody

Fig. 4.2b

Antibody responses of CBA/Ca males (O) and female (●) mice to ovine BLG.
Each point is the data from serum from a single mouse.



NDAb = no detectable antibody

previously shown, in Chapter 3, to produce good secondary antibody responses to ovine BLG.

i) The antibody responses of F1 BLG-transgenic mice to ovine BLG

The antibody data shown in Fig. 4.2a is from two experiments in which F1 BLG-transgenic male and female mice were challenged with ovine BLG. Following the first immunisation with ovine BLG, in the first experiment, no detectable anti-BLG IgG antibodies were found in the serum of male transgenic mice, and for 3/5 female transgenic mice. The remaining 2 female mice made very low titres of antibody. When this experiment was repeated F1 BLG-transgenic male and female mice made no detectable anti-BLG antibody.

Visual comparison of the anti-BLG IgG titres following primary immunisation with those titres following a secondary immunisation indicated that no significant increase in anti-BLG IgG titres occurred following challenging in both experiments (titre ranged from no detectable antibody to 2 for experiment 1 whilst the range was from no detectable antibody to 0 for experiment 2). Statistical confirmation of this observation was not carried out.

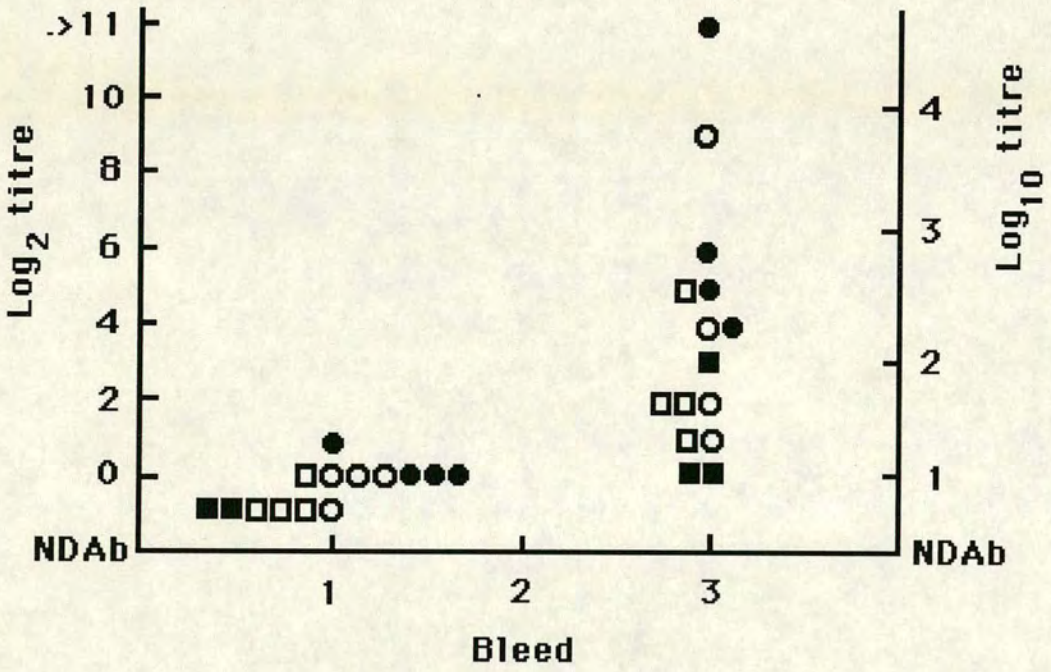
Following a third immunisation with ovine BLG male and female F1 BLG-transgenic mice, in experiment one, had anti-BLG titres (titre range 3-4) significantly greater than those detected after a second immunisation (titre range no detectable antibody to 2 ($p<0.01$ (males) and $p<0.005$ (females)). This small increase in antibody response was not detected in the repeat experiment.

ii) Comparing the antibody responses of BLG-transgenic and CBA/Ca mice, after immunisation with ovine BLG.

Although both CBA/Ca and F1 BLG-transgenic mice produced quite low anti-BLG titres (range 0 to 4 for CBA/Ca mice (see Fig.4.2b) and no detectable antibody to 0 for F1 BLG-transgenic mice (see Fig4.2a) following primary immunisation, the responses of F1 BLG-transgenic mice were slightly lower than

Fig. 4.3

Antibody responses of virgin and exbreeder CBA/Ca and F1 BLG-transgenic female mice to ovine BLG.
Each point is the data for serum from a single mouse.



□VIRGIN F1 BLG-TRANSGENIC FEMALES
■EX-BREEDER F1 BLG-TRANSGENIC FEMALES
○VIRGIN CBA/Ca FEMALES
●EX-BREEDER CBA/Ca FEMALES

NDAb= no detectable antibody

those of CBA/Ca mice. F1 BLG-transgenic mice also made a reduced immune response to ovine BLG compared to CBA/Ca mice following a second immunisation; the anti-BLG IgG titres produced by CBA/Ca male and female mice ranged from 2 to 12 (data from 2 experiments) whilst the anti-BLG IgG responses of F1 BLG-transgenic male and female mice ranged from no detectable antibody to 2 (data from 2 experiments). Statistical analyses of the pooled anti-BLG IgG responses, from each group of mouse, confirmed that F1 BLG-transgenic males and females made significantly lower responses than equivalent CBA/Ca mice ($p<0.0001$ (males) and $p<0.001$ (females)). This was also the case following the third immunisation ($p<0.001$ (males) and $p=0.0001$ (females)).
In conclusion, male and female BLG-transgenic mice appeared to be hyporesponsive, at the antibody level, to the protein product of the gene they possess. Comparison of the systemic anti-BLG IgG titres of transgenic mice and control CBA/Ca males and females indicated that both the primary and secondary IgG responses were affected.

iii) Antibody responses to ovine BLG in exbreeder female mice.
Exbreeder CBA/Ca and BLG-transgenic mice were immunised with ovine BLG to test whether pregnancy and lactation in female BLG-transgenic females would affect unresponsiveness at the antibody level as compared to virgin CBA/Ca and F1 BLG-transgenic controls. In this experiment the exbreeder mice had undergone three pregnancies.
The antibody titres following the first and third immunisations are shown in Fig. 4.3. No sera was collected following the secondary immunisation.
The absence of antibody production in 3/4 virgin F1 BLG-transgenic mice and 2/3 exbreeder F1 BLG-transgenic mice indicated that following a primary immunisation transgenic mice made little or no antibody responses to ovine BLG. CBA/Ca mice, in this experiment, also made low anti-BLG IgG titres following the first BLG immunisation.

Fig. 4.4 Cross-reactivity of anti-bovine and anti-ovine BLG antibodies

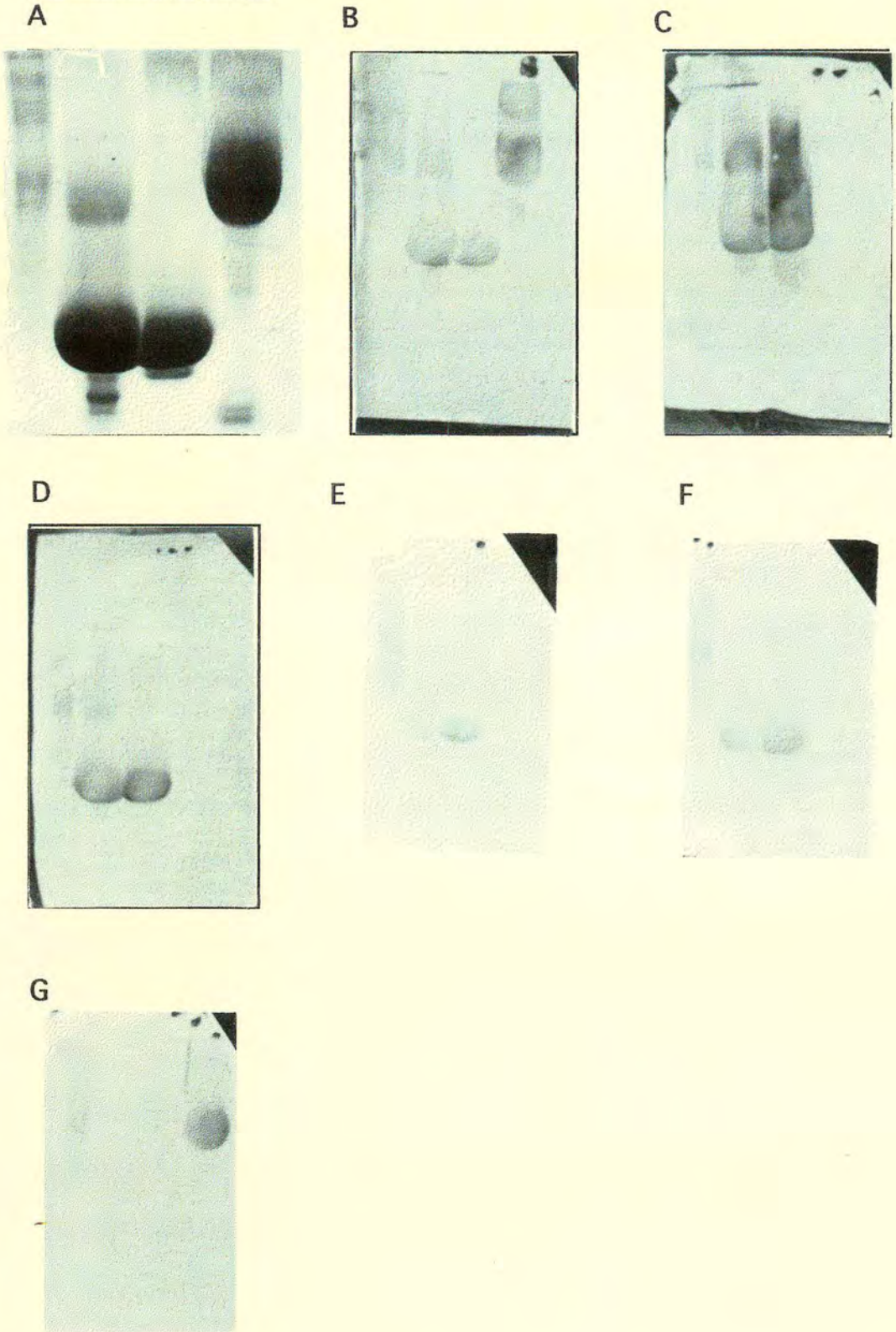


Fig. 4.4 Cross-reactivity of anti-ovine and anti-bovine BLG antibodies.

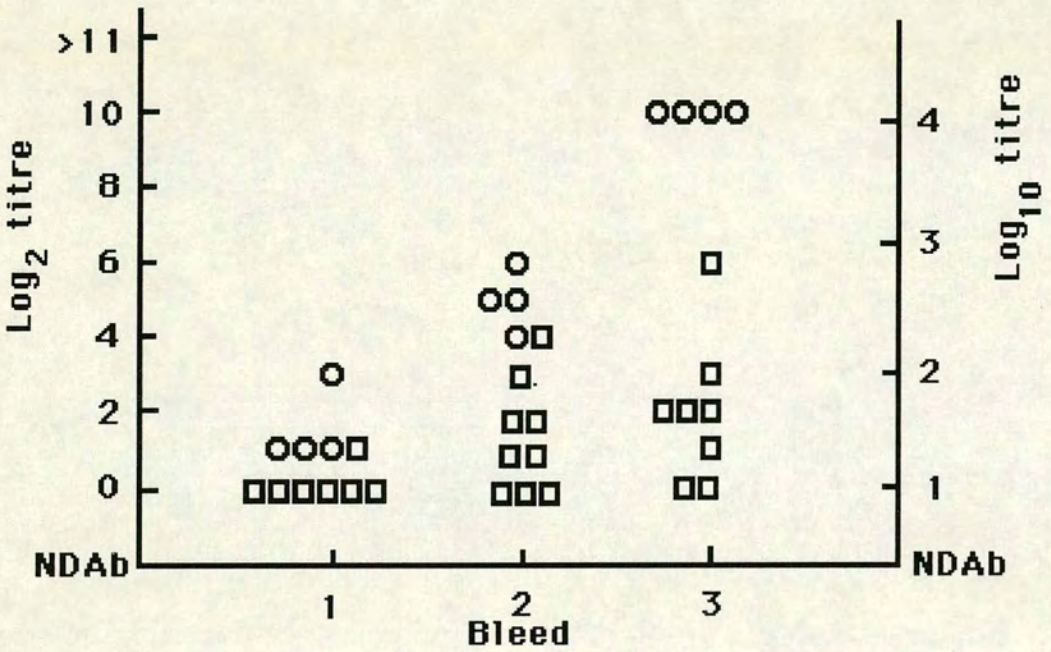
Gel A. Is a picture of a Coomassie Blue stained SDS Page gel containing in Lane 1 a prestained high molecular weight ladder (SIGMA), in Lane 2 bovine BLG (100mg/ml) (SIGMA, Poole, UK), in Lane 3 ovine BLG (31mg/ml) and in
Lane 4 OVA (50mg/ml) (SIGMA). 5μls of protein was added to 5μls of stacking buffer and 2μls of this mixture was added to the SDS Page gel.

Gels B-G are Western blots of similar SDS page gels incubated with different dilutions (see below) of anti-bovine, ovine or OVA antisera to detect cross-reactivity between reagents. Bands were visualised using a peroxidase linked second step antibodies.
Gel B. Rabbit anti-bovine BLG (1/200) (Nordic Immunological Laboratories, Tilburg, The Netherlands) and anti-rabbit IgG-HRP (1/500) (SAPU, Carluke, UK).
Gel C. Rabbit anti-ovine BLG (1/100) and anti-rabbit IgG-HRP (1/500) (SAPU).
Gel D. Pooled mouse anti-ovine BLG (1/500) from CBA/Ca mice immunised with ovine BLG and anti-mouse IgG-HRP (1/500) (SAPU).
Gel E. Pooled mouse anti-ovine BLG (1/10) from BLG-transgenic mice immunised with ovine BLG and anti-mouse IgG-HRP (1/500) (SAPU).
Gel F. Pooled mouse anti-ovine BLG (1/500) from non-transgenic mice immunised with ovine BLG and anti-mouse IgG-HRP (1/500) (SAPU).
Gel G. Pooled mouse anti-OVA from CBA/Ca mice immunised with OVA.

Results can be summarised as follows;
1) Gel B; antibodies against bovine BLG recognise bovine BLG and are cross-reactive with ovine BLG and OVA.
2) Gels C and D; antibodies against ovine BLG, raised in both rabbits and mice, react with ovine BLG and are cross-reactive with bovine BLG.
3) Gel E; antibodies against ovine BLG raised in BLG-transgenic mice recognise ovine BLG and are weakly cross-reactive with bovine BLG.
4) Gel F; antibodies against ovine BLG raised in non-transgenic mice recognise ovine BLG and are cross-reactive with bovine BLG.
5) Gel G; antibodies to OVA recognise OVA and not BLG.

Fig. 4.5

Antibody responses of CBA/Ca (O) and F1 BLG-transgenic (□) male mice to bovine BLG.
Each point is the data for serum from a single mouse.



NDAb = no detectable antibody

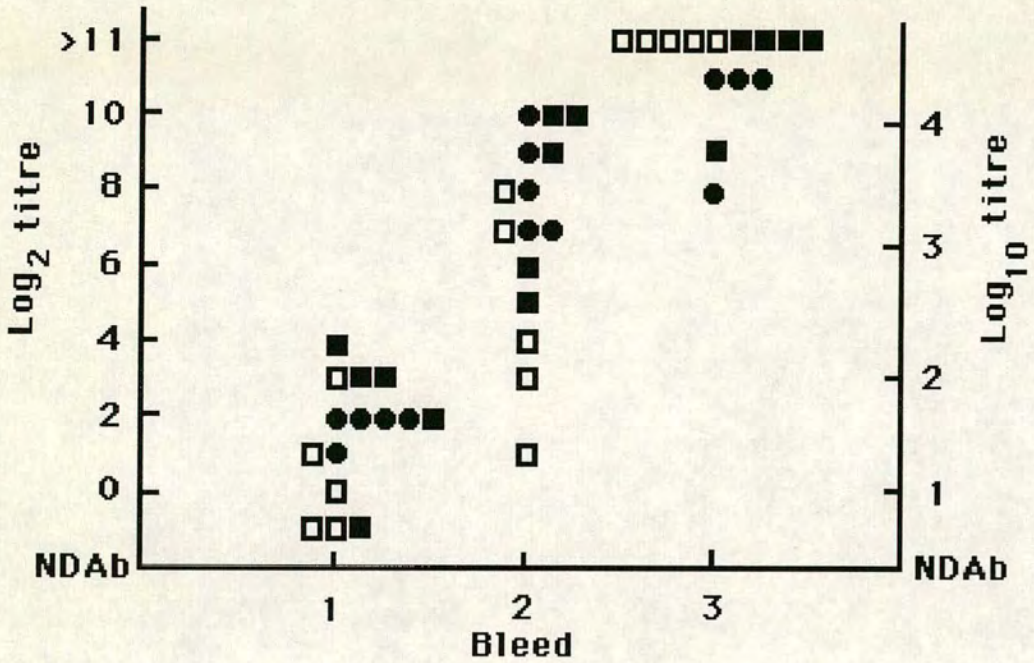
Following a third immunisation virgin and exbreeder F1 BLG-transgenic mice made equivalent anti-BLG responses. This was also found for the CBA/Ca virgin and exbreeder groups ($p<0.5$). Comparing the anti-BLG responses of CBA/Ca and BLG-transgenic exbreeder females confirmed that there was a significant difference ($p<0.05$) between these two groups of mice; transgenic mice made lower anti-BLG IgG titres than mice not possessing this gene.
These data suggest that pregnancy did not affect the antibody hyporesponsiveness to ovine BLG seen in BLG-transgenic mice.

iv) The antibody responses of F1 BLG-transgenic and CBA/Ca male mice to bovine BLG

Bovine and ovine BLG are immunologically cross-reactive as shown via Western blotting analysis (see Fig.4.4). Bovine and ovine BLG were electrophoresed on a 15% polyacrlyamide gel and Western blotted. Detecting antibodies used were derived from the pooled sera of non-transgenic and transgenic male and female mice immunised with ovine BLG as well as rabbit anti-bovine BLG and rabbit 669 anti-ovine BLG. Data from the Western blotting analysis showed that antibodies to bovine and ovine BLG were cross-reactive (Fig. 4.4). It was thus predicted that mice possessing the ovine BLG gene would be tolerant to bovine BLG as well as ovine BLG.
To test this hypothesis F1 BLG-transgenic male (no female mice were analysed) were immunised with bovine BLG and the anti-bovine BLG IgG responses, following each immunisation, analysed using a bovine BLG specific ELISA. CBA/Ca male mice were also immunised with bovine BLG. Antibody responses are shown in Fig. 4.5.
CBA/Ca males responded to a primary immunisation with bovine BLG by producing a low level of anti-BLG IgG antibody (titres ranged from 1-3). Male and female F1 BLG-transgenic mice also produced a low antibody response (titre ranged from no detectable antibody - 1). Immunological memory in CBA/Ca mice was generated, indicated by the significant increase in antibody titres following a second (range of 4-6, $p<0.05$) and

Fig. 4.6

Antibody responses of CBA/Ca (●), F1 BLG-transgenic (■) female mice and F1 BLG-transgenic male (□) mice to OVA. Each point is the data for serum from a single mouse.



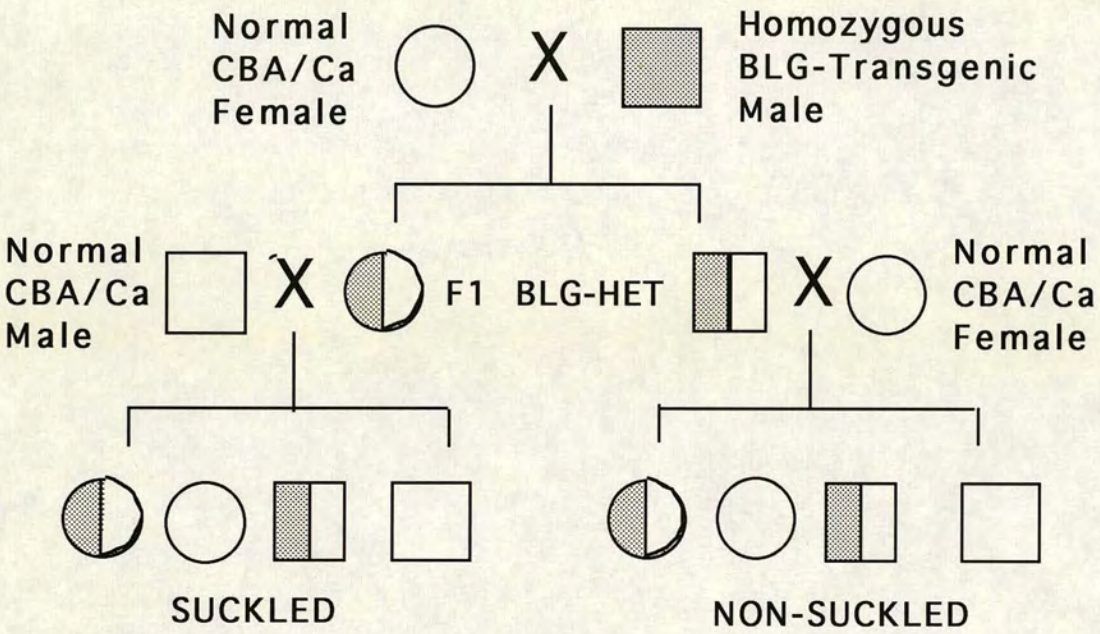
NDAb = no detectable antibody

third immunisation (titres of 10, $p<0.05$). In contrast, this did not occur for the F1 BLG-transgenic mice.
F1 BLG-transgenic male mice also made significantly lower antibody response to bovine BLG than the controls following each of the three immunisations ($p<0.01$ (1st), $p<0.005$ (2nd and 3rd)). (Although female mice were not included in this experiment it is predicted that the BLG-transgenic female would have a reduced response to bovine BLG as compared to female CBA/Ca mice). In conclusion, possession of the ovine BLG-transgene results in unresponsiveness to bovine BLG as well as ovine BLG following both primary and secondary immunisations compared to non-transgenic mice.

v) The antibody responses of F1 BLG-transgenic and CBA/Ca mice to Ovalbumin

Following a first immunisation with OVA, CBA/Ca females (no CBA/Ca males were tested) and F1 BLG-transgenic male and female mice produced low anti-OVA specific responses; the antibody responses ranged from a titre of 1-2 for CBA/Ca mice, and from no detectable antibody to 4 for transgenic mice, see Fig. 4.6. High anti-OVA IgG titres were observed following a second immunisation with OVA in all groups of mice, as compared to the primary antibody response ($p<0.01$ (CBA/Ca) $p<0.05$ (transgenic males) and $p<0.01$ (transgenic females)). Secondary anti-OVA IgG responses of F1 BLG-transgenic female mice were comparable to those of CBA/Ca mice. A further increase in the anti-OVA response for all groups of mice occurred after a third immunisation ($p<0.05$ for CBA/Ca, transgenic males and transgenic females) and the tertiary anti-OVA responses of CBA/Ca and transgenic females were comparable.
In conclusion, F1 BLG-transgenic female mice do not appear hyporesponsive to the control antigen OVA and although no comparison between the anti-OVA response of CBA/Ca and F1 BLG-transgenic males was possible these transgenic mice also appear responsive to this protein.

Fig. 4.7 Mating strategy



4.2) Heterozygous BLG male X CBA/Ca females (non-BLG-suckled) vs CBA/Ca male X Heterozygous BLG female (BLG-suckled)

The second group of transgenic and non-transgenic mice tested for antibody responsiveness to ovine BLG were derived from mating F1 heterozygous BLG-transgenic mice with CBA/Ca partners in such a way that offspring were either exposed to BLG via milk produced from lactating transgenic females or they suckled on "normal" CBA/Ca mouse milk. These crosses were set up to investigate whether suckling BLG-containing milk would induce oral tolerance. The mating protocol is illustrated in Fig. 4.7.

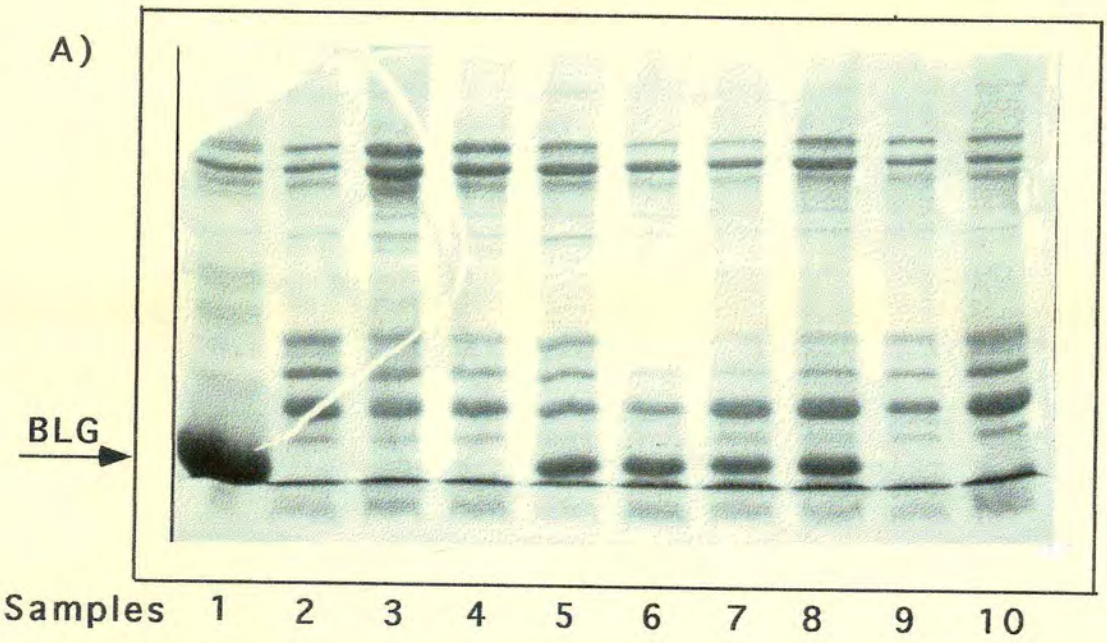
To ensure that lactating BLG-transgenic females produced milk containing the ovine BLG protein and that it was being passed to suckling pups, a few mice suckling BLG-containing or "normal" milk were killed, stomach contents run on an SDS gel and any BLG present identified, using rabbit 669 anti-ovine BLG, following Western blotting. A known ovine BLG sample (sheep's milk) was included. 9/9 mice suckled on BLG-containing milk had BLG protein present within their stomachs following feeding whilst 5/5 mice suckled on "normal" mouse milk did not, see Fig. 4.8.

After the pups had suckled for 21 days they were weaned and placed on a diet known to contain bovine BLG. At 3 months of age, adult mice were identified as transgenic or non-transgenic, and immunised with ovine BLG or OVA as described in Chapter 2. Sera were collected at various intervals and anti-ovine IgG or anti-OVA IgG titres were estimated for each mouse, as before.

i) The antibody responses of BLG-transgenic and non-transgenic mice to ovine BLG.

Three separate experiments were conducted. The first two experiments (represented by triangles and squares, respectively, in Figs 4.9-4.12) only had a few mice per group such that no statistical comparison between groups was carried out. The third experiment (represented by circles in

Fig. 4.8. Testing the milk of a lactating BLG-transgenic mouse for the presence of ovine BLG.



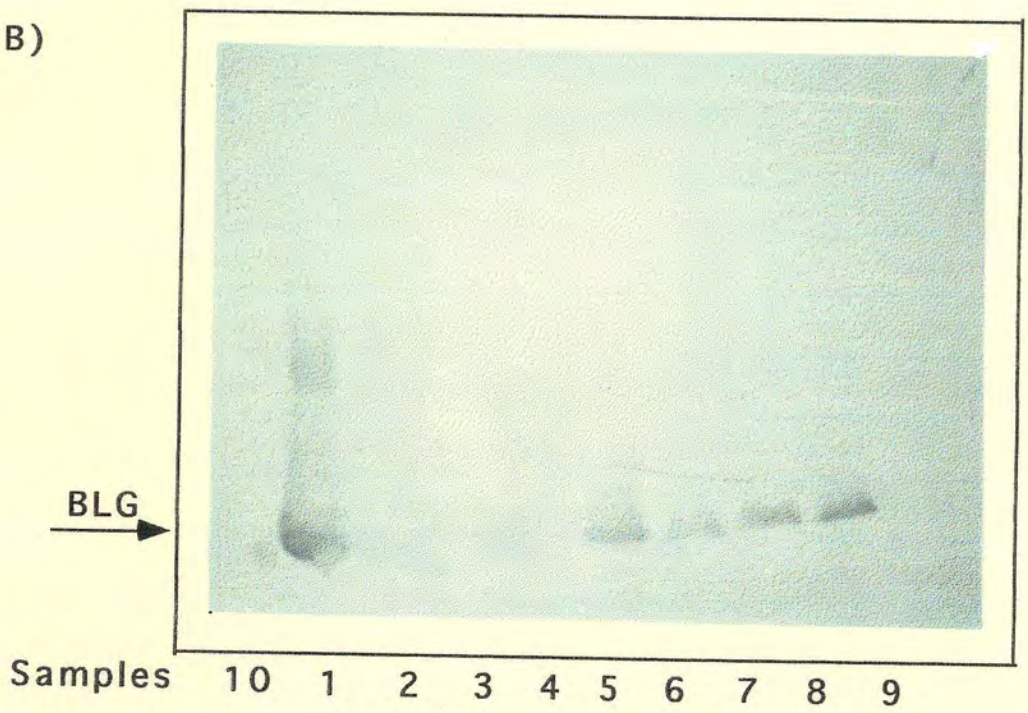


Fig. 4.8. Testing the milk of a lactating BLG-transgenic female mice for the presence of ovine BLG.

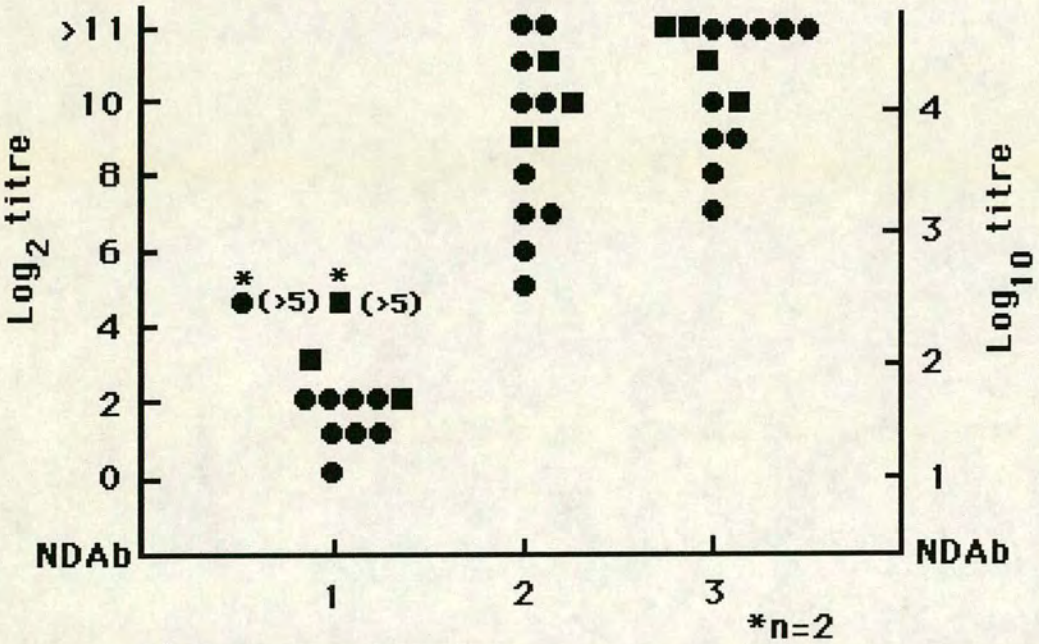
A) Picture of a Coomassie blue stained SDS Page gel containing the stomach contents of four 7-10 day old pups from a lactating BLG-transgenic female (samples 5, 6, 7 and 8) and five 7-10 day old pups from a lactating non-transgenic female (samples 2, 3, 4, 9 and 10). Sample 1 is ovine BLG purified from sheeps milk.
Pups were killed and their stomachs contents removed. 50μls of PBS was added to each of the stomach contents and following homogenising 5μls of this mixture was added to 5μls of PBS plus 10μls of sample buffer. 2.5μls of this mixture was then added to a 15% SDS Page gel.

B) Picture of a Western blot of a similar SDS Page gel.
The detecting antibodies used in this Western Blot were rabbit anti-ovine BLG. Second step antibodies were anti-rabbit-HRP. Positive BLG bands were visualised using a peroxidase detecting system.

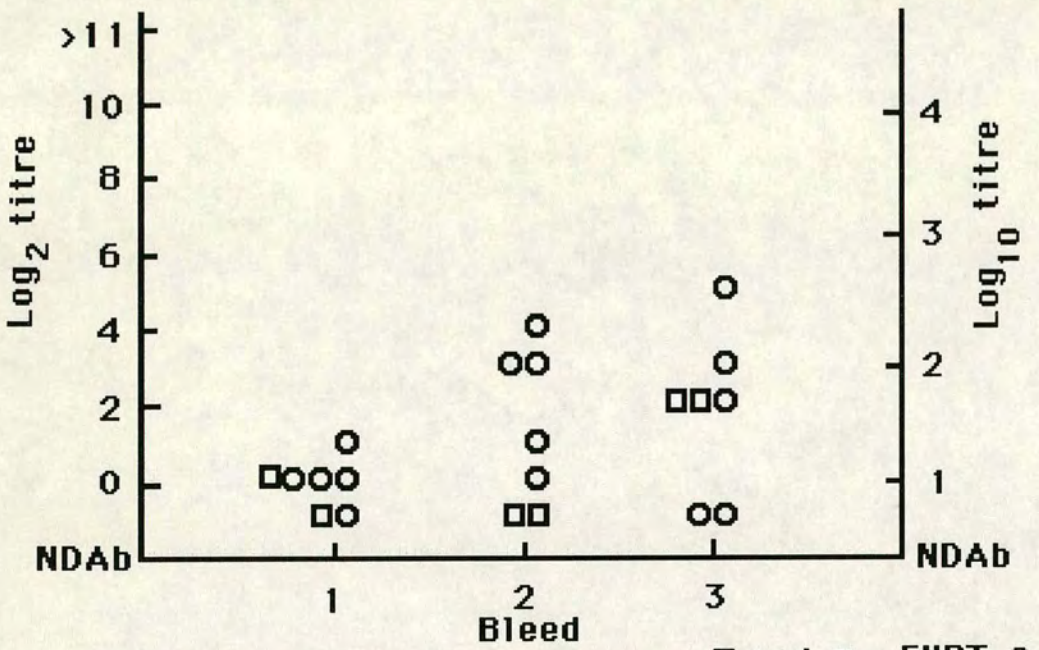
Fig 4.9

Antibody responses of female mice suckled on BLG-containing milk to ovine BLG. Each point is the data for serum from a single mouse.

a) Non-transgenic mice



b) Transgenic mice



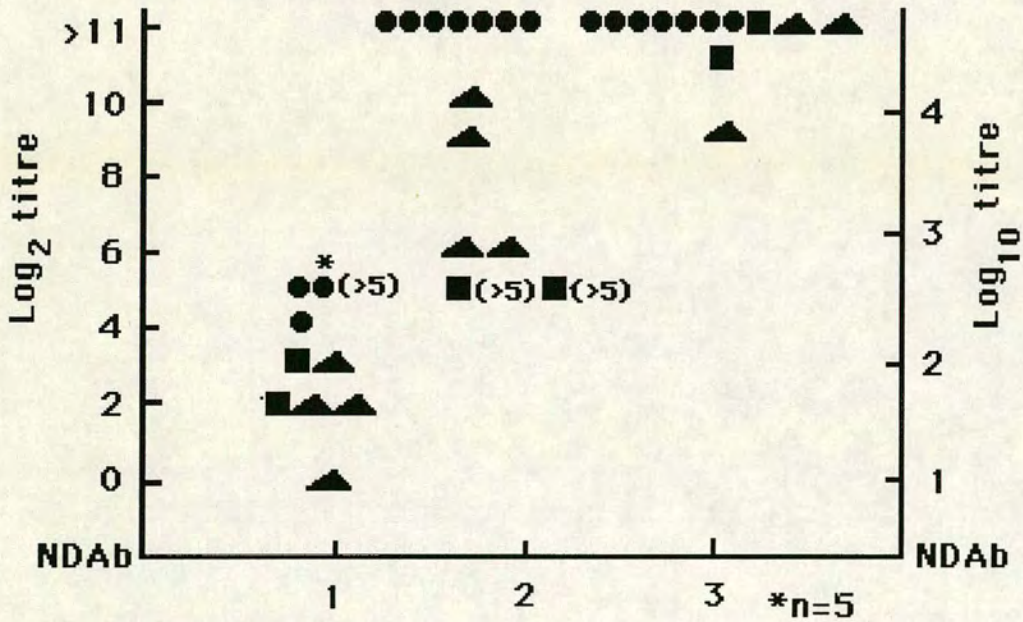
Data represents 2 experiments; □ and ■ = EXPT 2
○ and ● = EXPT 3

NDAb = no detectable antibody

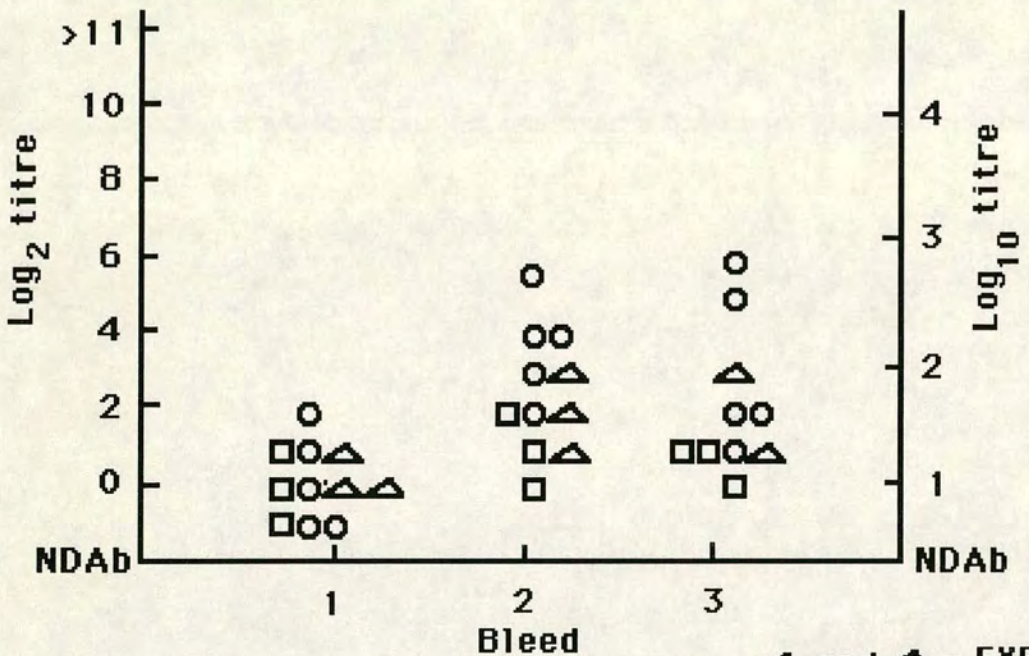
Fig 4.10

Antibody responses of female mice suckled on "normal" mouse milk to ovine BLG. Each point is the data for serum from a single mouse.

a) Non-transgenic



b) Transgenic



Data represents 3 experiments; ▲ and △ = EXPT 1, ■ and □ = EXPT 2, ● and ○ = EXPT 3

NDAb = no detectable antibody

the aforementioned figures) however did allow statistical analysis of groups of mice and the p values given represent those from analysis of this experiment. In the following sections the anti-BLG responses of BLG-transgenic mice compared with their non-transgenic littermates are discussed.

a) Female mice suckled on BLG-containing milk (Fig. 4.9).
The antibody data in Fig. 4.9 represents data from experiments 2 and 3. Although experiment 2 contained only a few mice, it was noted that there was an obvious difference between transgenic and non-transgenic individuals' anti-ovine BLG IgG titres following secondary immunisation; transgenic mice made lower antibody titres to BLG than non-transgenic mice.
This was also the case for mice in experiment 3, although following a secondary immunisation transgenic mice made significantly greater ($p<0.05$) antibody responses to BLG than following a primary immunisation; both the primary and secondary anti-BLG IgG titres were significantly lower than their non-transgenic littermates ($p<0.02$ and $p<0.01$, respectively). This was also the case following a third immunisation ($p<0.01$).

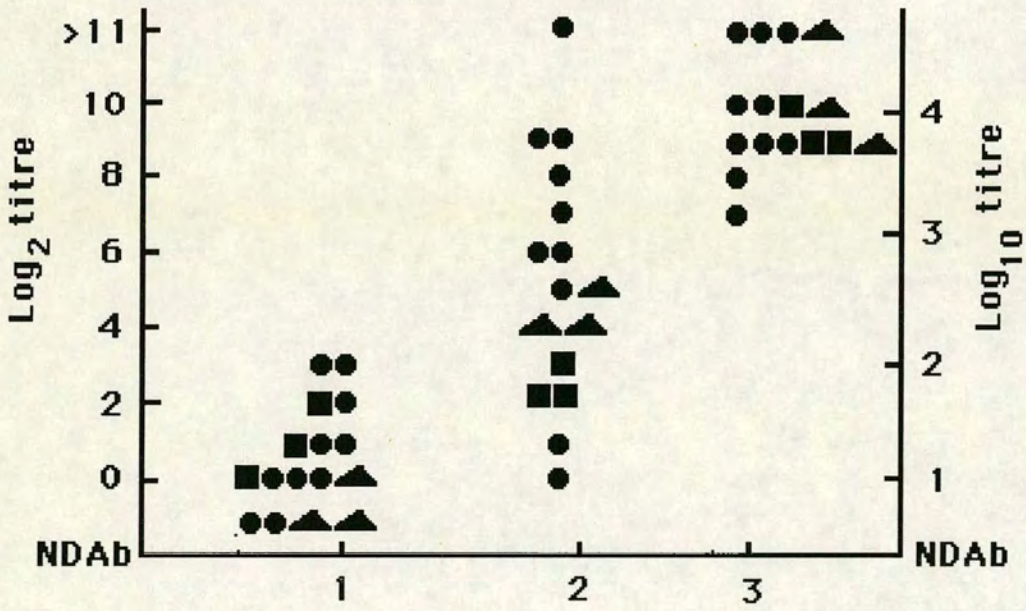
b) Female mice suckled on "normal" mouse milk (Fig. 4.10)
Figure 4.10 contains data from all three experiments. Each experiment showed that there was an obvious difference between transgenic and non-transgenic mice, again transgenic mice made lower antibody responses than non-transgenic mice following each immunisation.
The primary responses of transgenic mice in this experiment were comparable to the responses of non-transgenic mice. A significant increase ($p<0.05$) in the anti-BLG response occurred in transgenic mice following a secondary immunisation however these were significantly reduced compared to the non-transgenic mice ($p<0.001$). This was also found after a third immunisation ($p<0.001$).

c) Male mice suckled on BLG-containing milk (Fig.4.11)

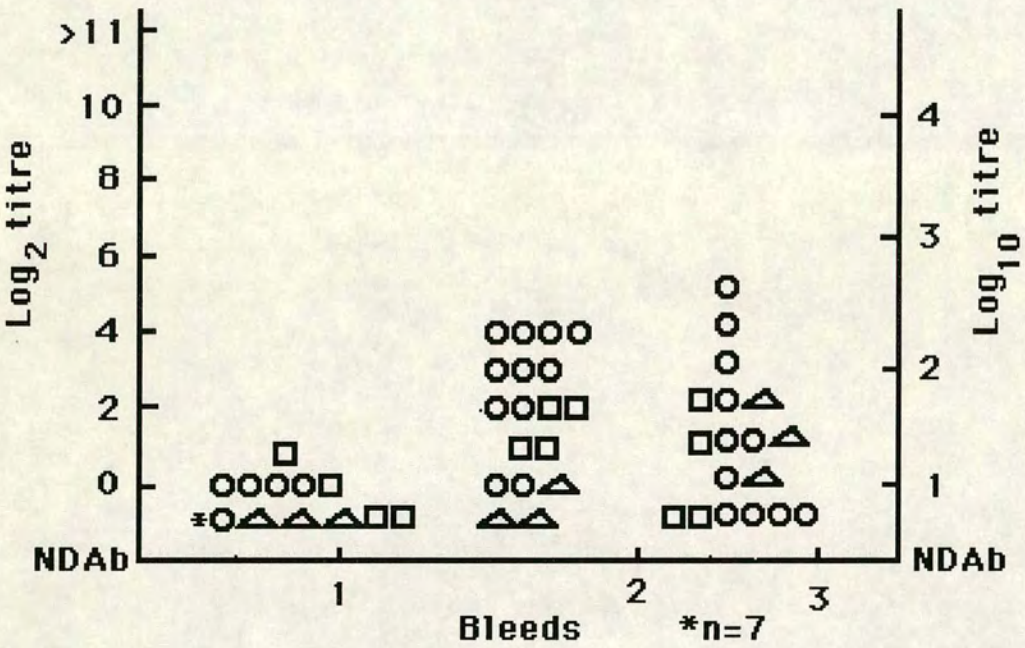
Fig. 4.11

Antibody responses of male mice suckled on BLG-containing milk to ovine BLG. Each point is the data for serum from a single mouse.

a) Non-transgenic



b) Transgenic



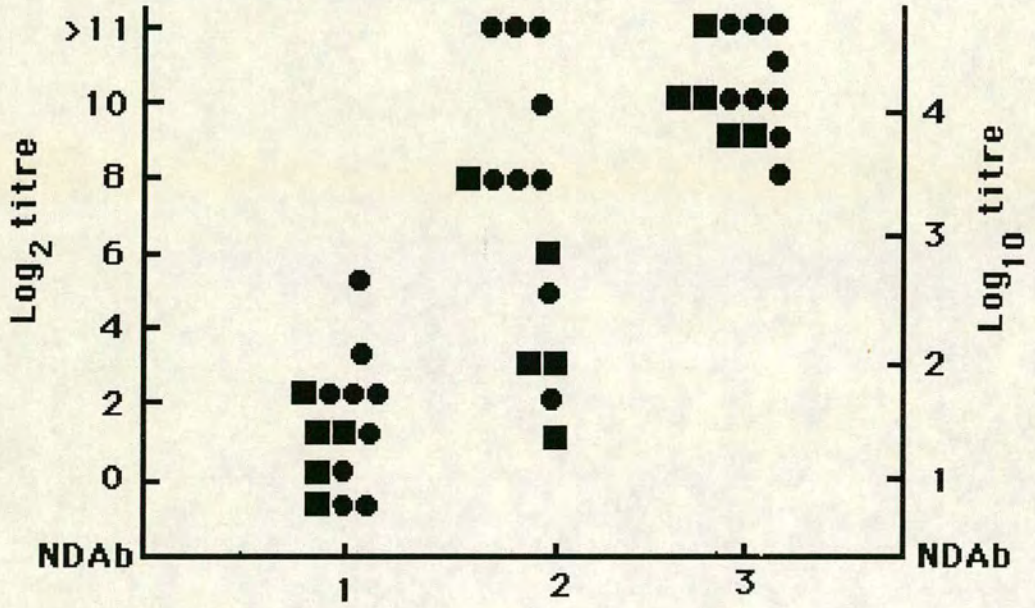
Data represents 3 experiments; ▲ and △ = EXPT 1
■ and □ = EXPT 2
● and ○ = EXPT 3

NDAb = no detectable antibody

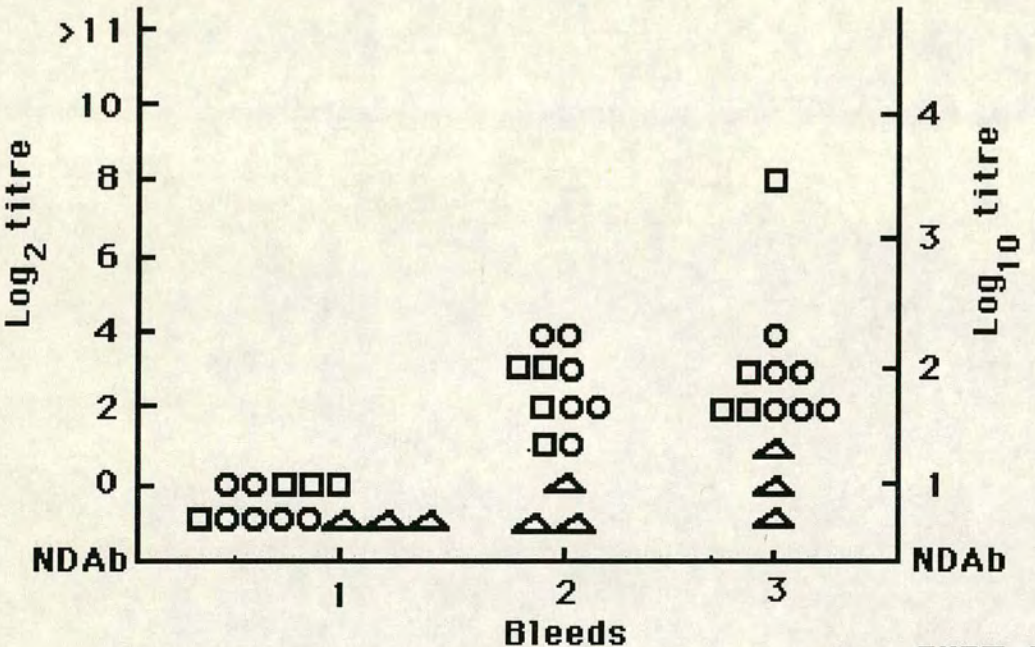
Fig. 4.12

Antibody responses of male mice suckled on "normal" mouse milk to ovine BLG. Each point is data for serum from a single mouse.

a) Non-transgenic



b) Transgenic



Data represents 3 experiments; △ = EXPT 1, ■ and □ = EXPT 2, ● and ○ = EXPT 3

NDAb = no detectable antibody

Figure 4.11 contains data from three experiments and as for female BLG-transgenic mice, male BLG-transgenic mice had obviously lower anti-BLG IgG titres than their non-transgenic littermates. However, in the first two experiments a convincing difference between these mice only occurred following a third immunisation. The reason for this is unknown. In the third experiment, transgenic mice made a significant lower anti-BLG responce following both the second ($p<0.01$) and third ($p<0.001$) immunisations as compared to non-transgenic mice.

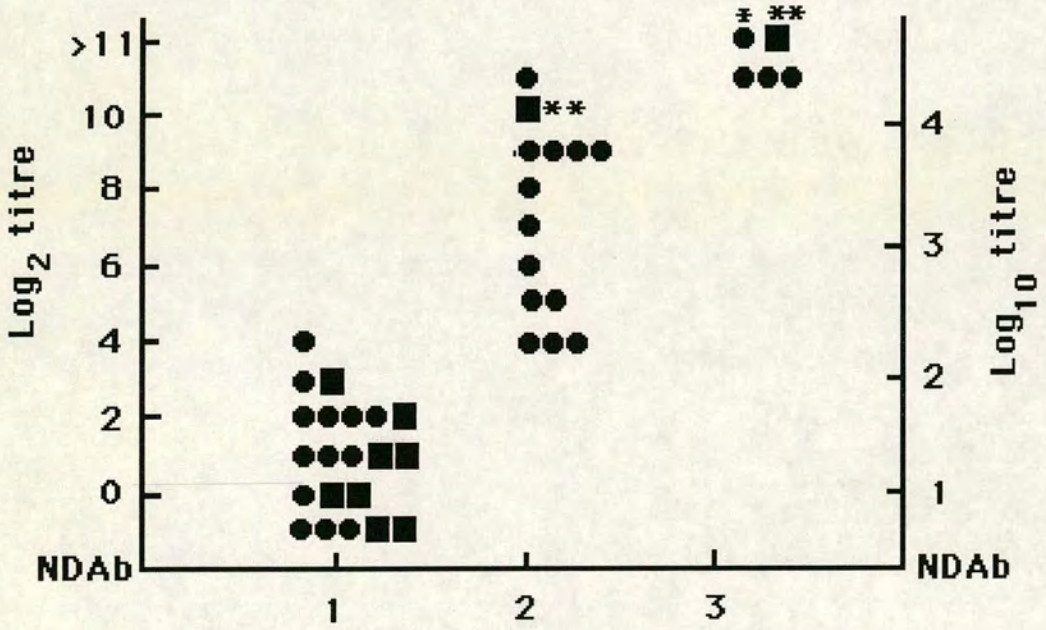
d) Male mice suckled on "normal" mouse milk (Fig. 4.12)
Comparing BLG-transgenic and non-transgenic mice from experiments 2 and 3, no non-transgenic mice were analysed in experiment 1, also indicated that BLG-transgenic mice made lower anti-BLG IgG responses following a second ($p<0.01$, experiment 3) and third ($p<0.001$, experiment 3) immunisation than non-transgenic littermates. No significant difference was found following a primary immunisation.

e) Comparing the anti-BLG IgG responses of mice suckled on either BLG-containing or "normal" mouse milk.
When non-transgenic females from the “suckled” and “non-suckled” groups in the third experiment were compared a significant difference, following each immunisation, was observed; exposure to BLG from birth appeared to reduced BLG-specific IgG levels ($p<0.05$, $p<0.005$ and $p<0.05$ when the primary, secondary and tertiary titres were compared). It should be stated that although IgG titres were reduced, antibody levels never fell to those of their transgenic littermates and some mice appeared unaffected by exposure to BLG, making high anti-BLG IgG titres.
Since the antibody responses to BLG from each of the three experiments was comparable the data was pooled to further check if the difference seen above (i.e. non-transgenic mice suckled on BLG-containing milk having reduced antibody responses to BLG) remained. The primary anti-BLG IgG

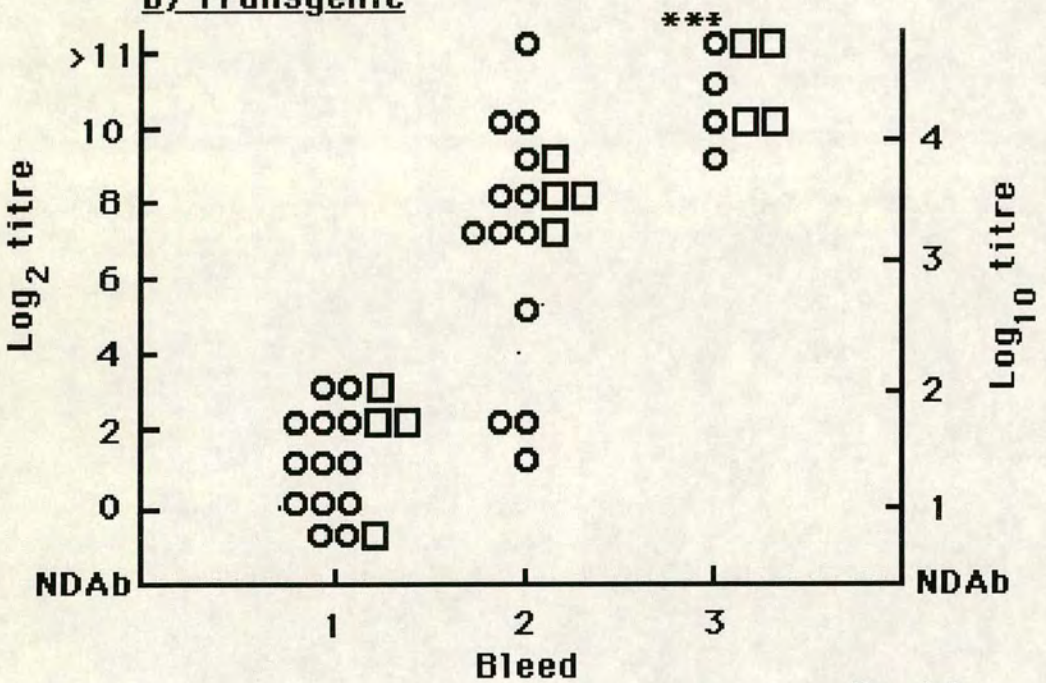
Fig 4.13

Antibody responses of male (circles) and female (squares) mice suckled on BLG-containing milk to OVA.
Each point is the data for serum from a single mouse.

a) Non-transgenic



b) Transgenic



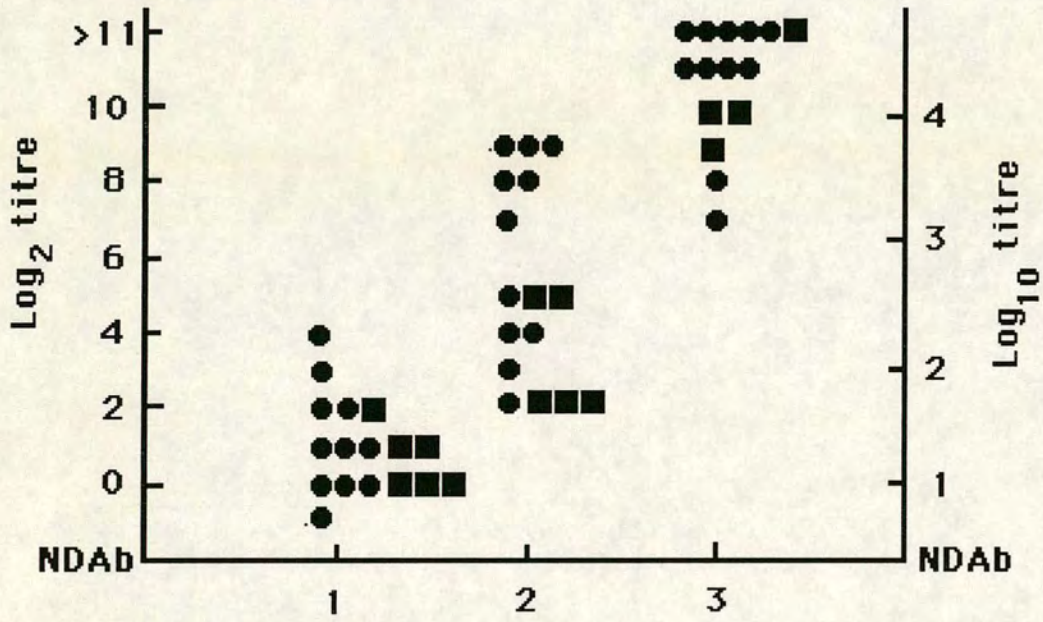
*n=10
**n=8
***n=10

NDAb = no detectable antibody

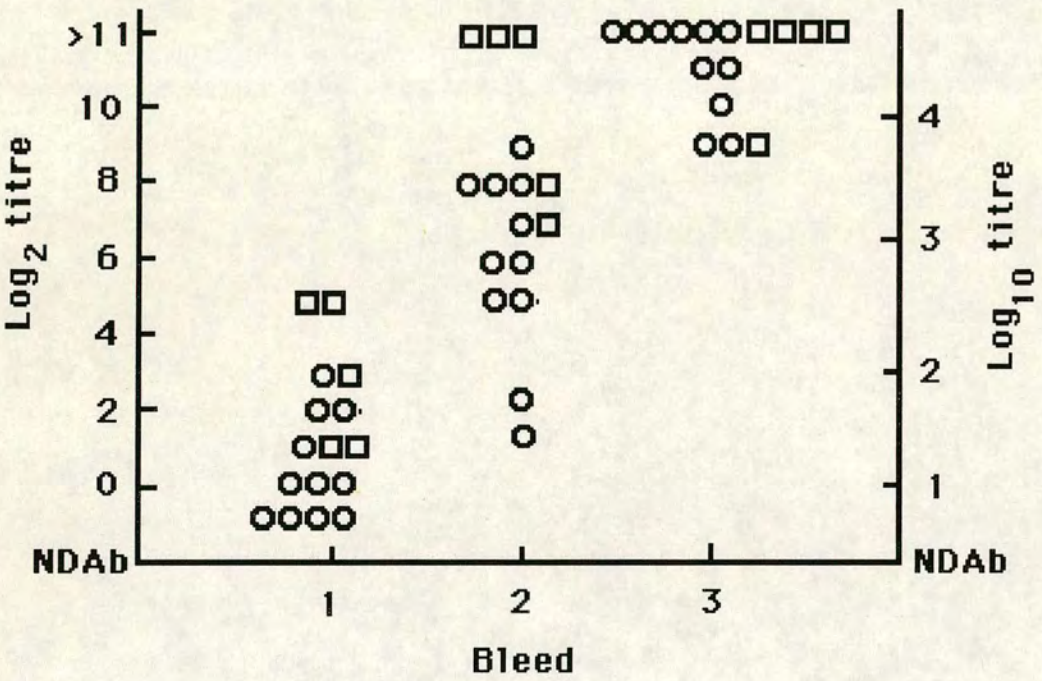
Fig 4.14

Antibody responses of male (circles) and female (squares) mice suckled on "normal" mouse milk to OVA.
Each point is the data for serum from a single mouse

a) Non-transgenic



b) Transgenic



NDAb = no detectable antibody

responses of non-transgenic female mice suckled on BLG-containing milk was significantly reduced as compared to those suckled on normal mouse milk ($p<0.05$). However no significant reduction in the anti-BLG response was evident after both a second and third immunisation.

Suckling on BLG-containing milk did not result in reduced anti-BLG IgG responses to this protein in non-transgenic male mice; the anti-BLG IgG responses of non-transgenic males suckled on either BLG-containing or "normal" mouse milk were comparable after each immunisation. This was also the case after the data from each experiment was pooled.

Comparing the anti-BLG IgG responses, from the third experiment, of transgenic mice suckled on BLG-containing milk with those of transgenic mice suckled on "normal" mouse milk revealed that the anti-BLG IgG responses of these two groups of mice following any of the challenges were comparable. This was also the case after the data was pooled. These data suggest that; 1) suckling on BLG-containing milk did not induce oral tolerance to Ovine BLG and 2) exposure to BLG-containing milk was not responsible for the reduced anti-BLG IgG responses seen for mice carrying the BLG transgene

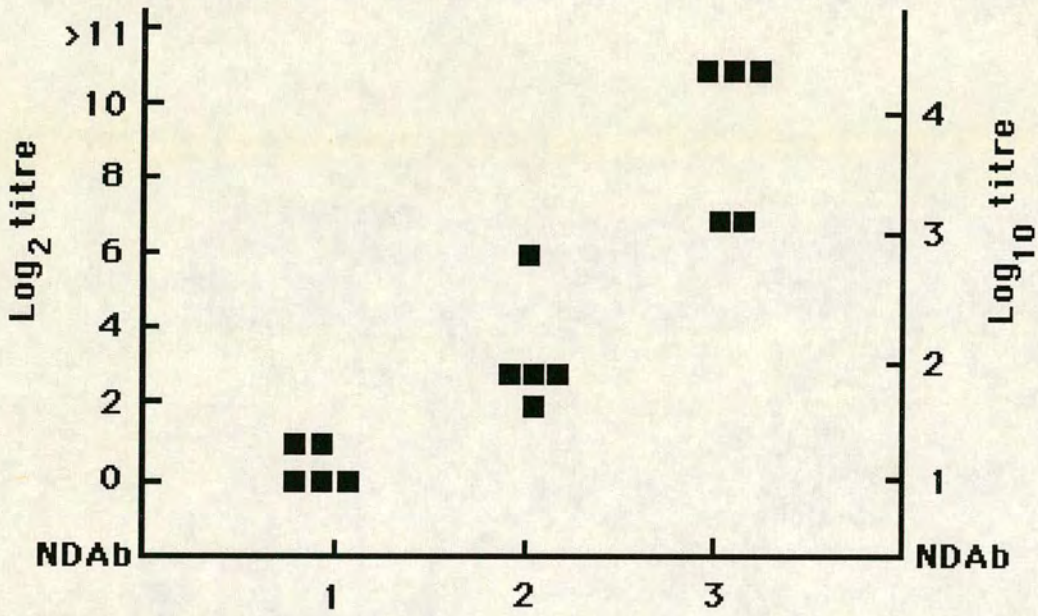
ii) The antibody responses of female and male transgenic mice to Ovalbumin

To investigate whether BLG-transgenic mice were hyporesponsive to other proteins these mice were immunised with an unrelated protein, OVA, and the anti-OVA IgG responses of BLG-transgenic mice were compared with the anti-OVA IgG responses of non-transgenic littermates. Despite the wide range in anti-OVA IgG titres (see Figs 4.13 and 4.14) both BLG-transgenic and non-transgenic male and female mice were obviously capable of responding to OVA. The antibody responses of BLG-transgenic and non-transgenic mice were equivalent. These data are similar to those shown for F1 BLG-transgenic mice in Fig. 4.6.

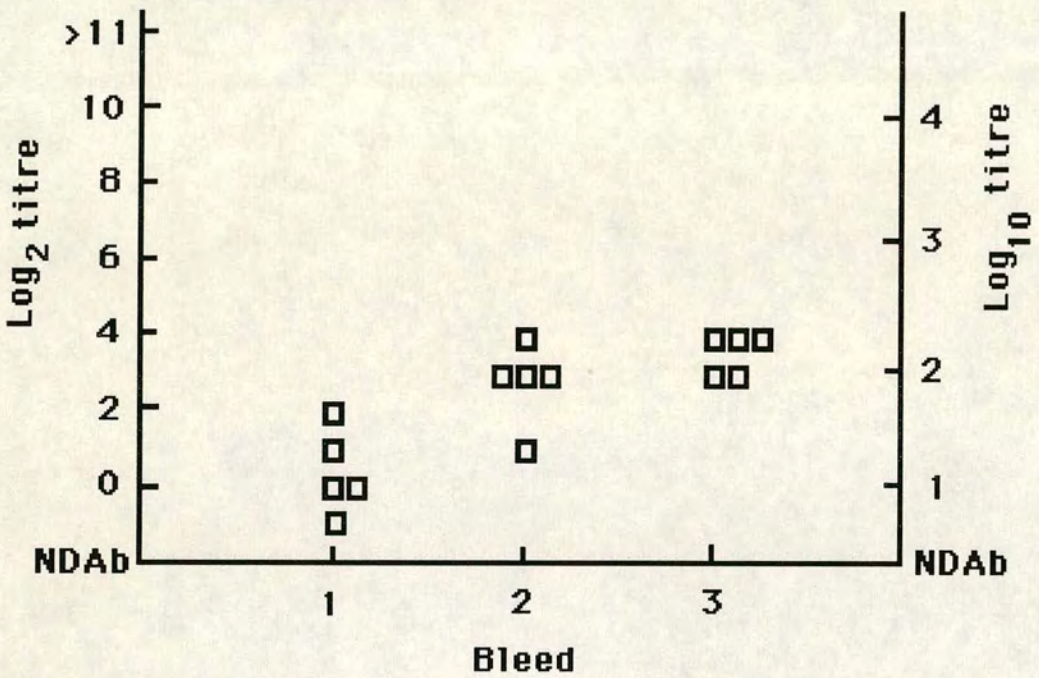
Fig. 4.15

Antibody responses of G6 female mice suckled on BLG-containing milk to ovine BLG
Each point is the data for serum from a single mouse.

a) Non-transgenic



b) Transgenic

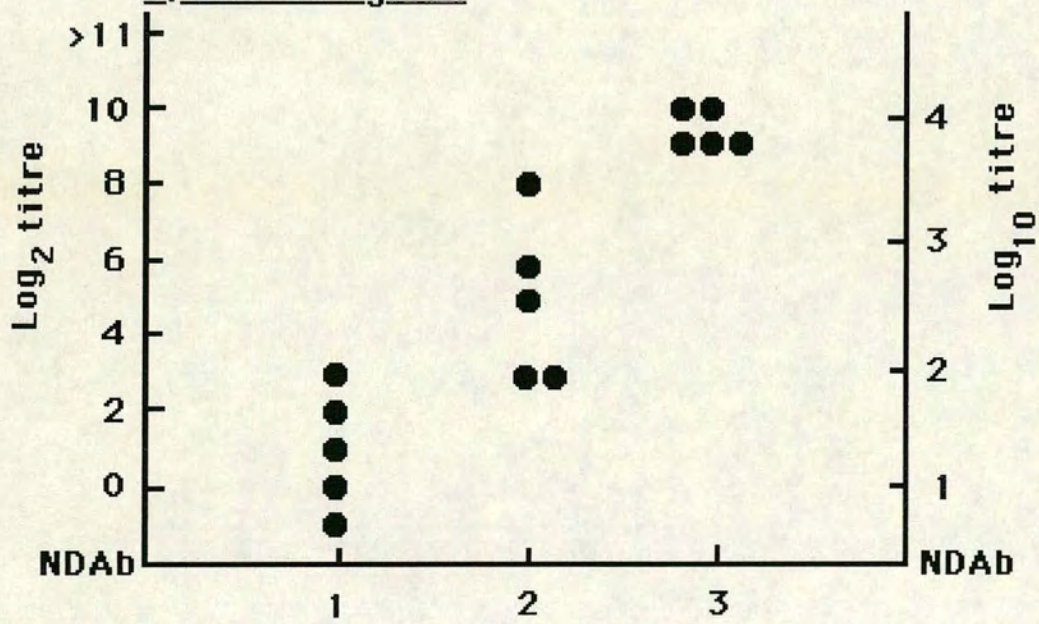


NDAb = no detectable antibody

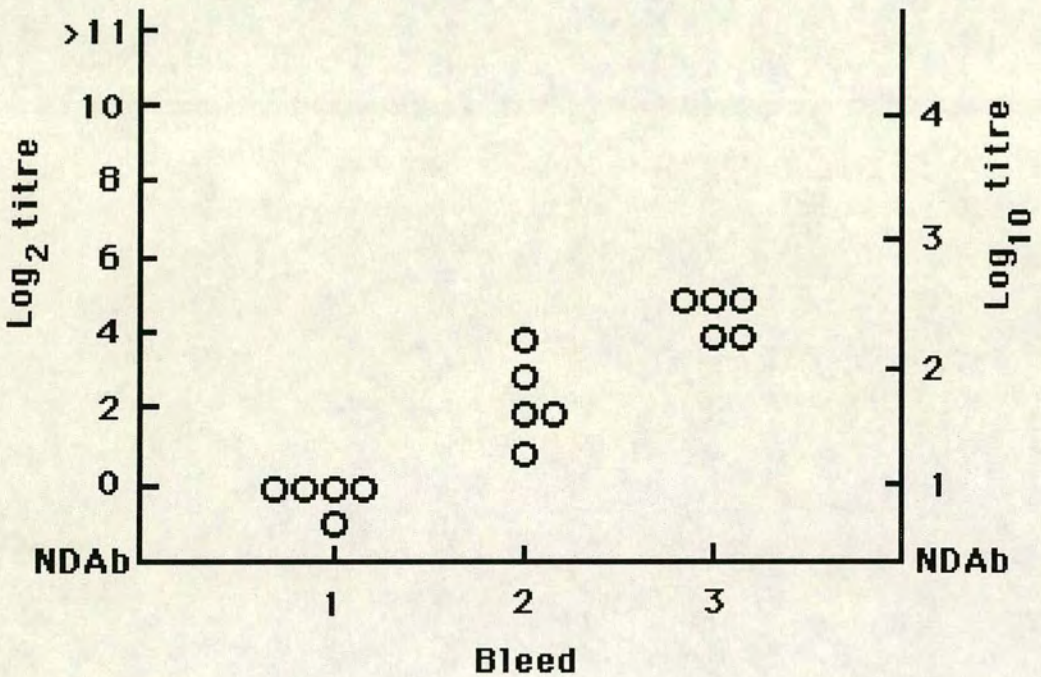
Fig. 4.17

Antibody responses of G6 males suckled on BLG-containing milk to ovine BLG. Each point is data for serum from a single mouse.

a) Non-trangenic



b) Transgenic



NDAb = no detectable antibody

It was also noted that suckling BLG-containing milk did not interfere with the ability of transgenic or non-transgenic mice to respond to OVA.

iii) The antibody responses of mice fed a diet lacking whey protein to ovine BLG .

F1 BLG-transgenic mice and CBA/Ca mice were placed on a diet lacking whey protein following weaning and at 8 weeks of age these mice were mated as described in Fig. 4.7. Following weaning offspring from these crosses were also placed on a diet lacking bovine BLG. This experiment was set up to investigate whether bovine BLG in the diet interfered with the development of tolerance to ovine BLG. The antibody responses are not shown here but can be found in Appendix 10.

The data indicated that the presence or absence of whey protein in the diet did not affect the observed hyporesponsiveness in transgenic mice nor the development of oral tolerance; adult BLG-transgenic mice mice exposed to BLG, in their mother's milk, during the first 21 days of life were not rendered orally tolerant to this antigen.

4.3) Generation 6 (G6)-backcrossed mice.

During the backcrossing of the BLG transgene onto a CBA/Ca background, G5 mice were crossed with CBA/Ca mice such that the resulting offspring either suckled on BLG-containing milk or on "normal" mouse milk. At three months of age these G6 offspring were indentified as BLG-transgenic or non-transgenic via PCR and then immunised with ovine BLG as previously described. This experiment was set up to eliminate any possible background genetic effects.

i) The antibody responses of G6 mice to ovine BLG (Figs 4.15 to 4.**18**)

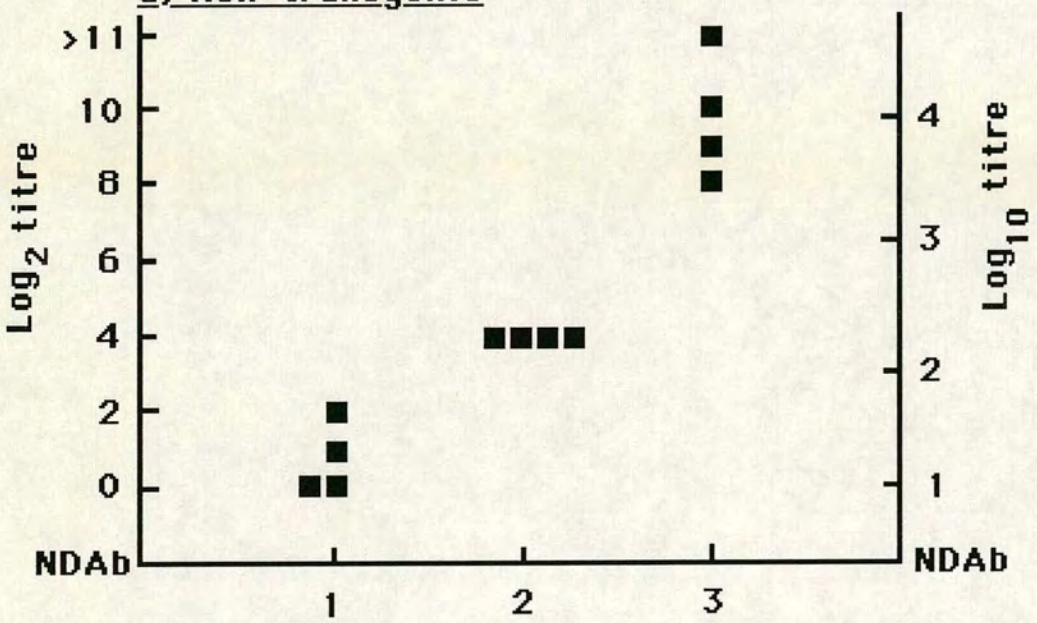
a) Female and male mice suckled on BLG-containing milk (Figs 4.15 and 4.17).

BLG-transgenic and non-transgenic G6 male and female mice suckling on BLG-containing milk produced anti-BLG IgG

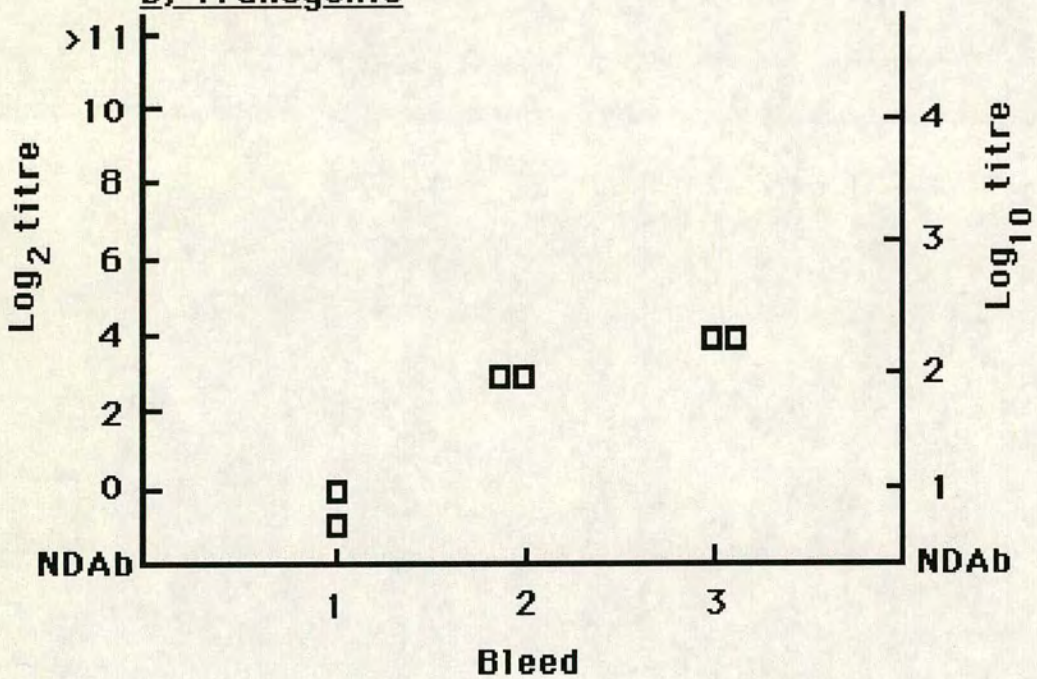
Fig. 4.16

Antibody responses of G6 female mice suckled on "normal" mouse milk to ovine BLG.
Each point is the data for serum from a single mouse.

a) Non-transgenic



b) Transgenic

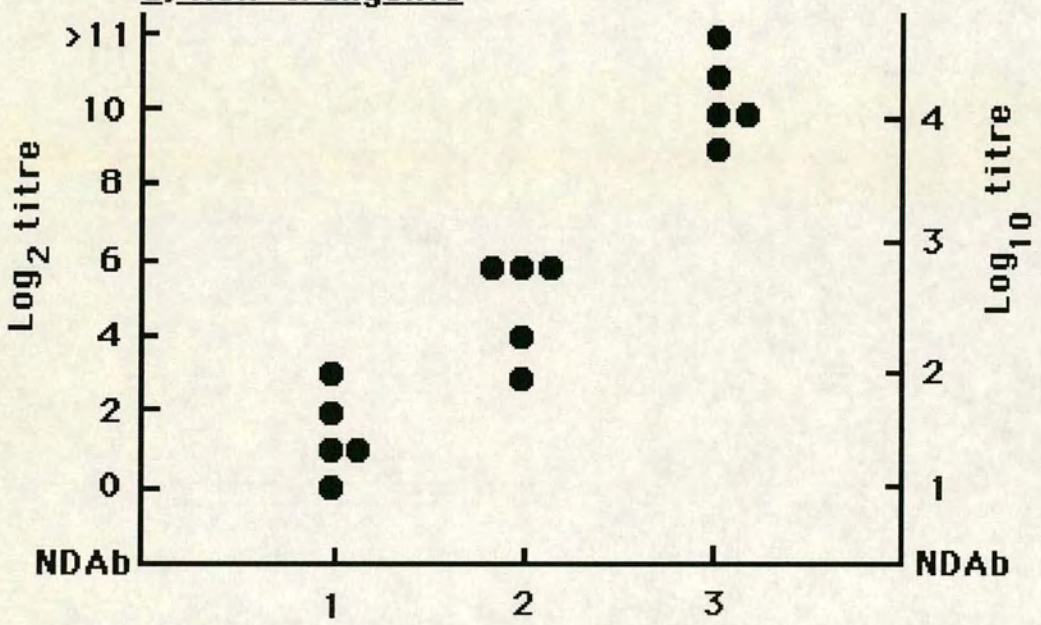


NDAb = no detectable antibody

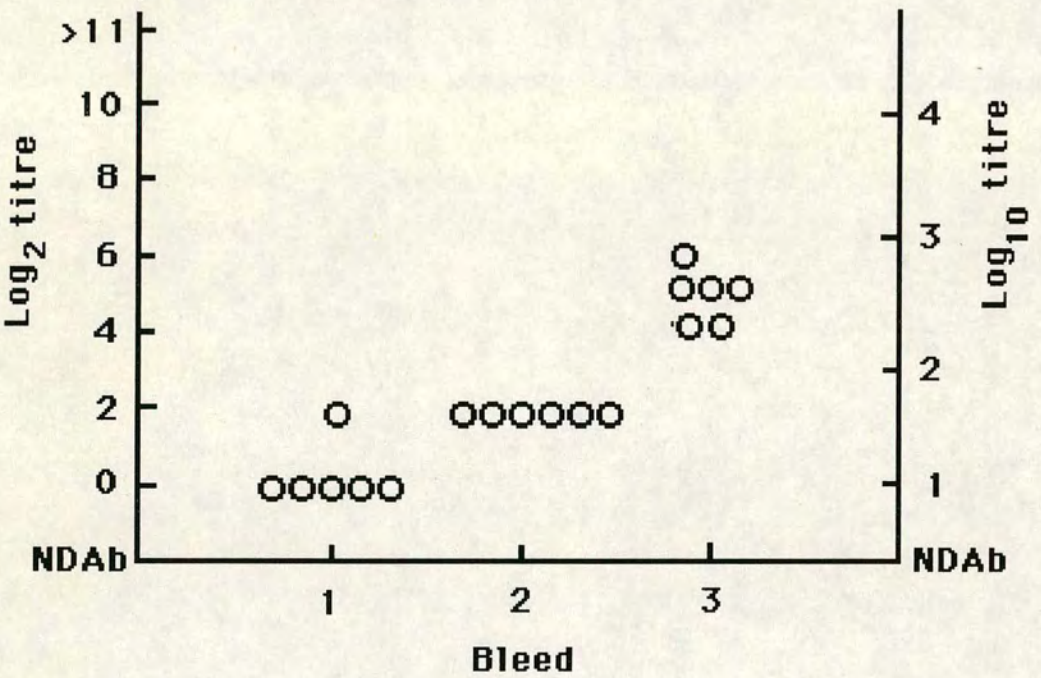
Fig. 4.18

Antibody responses of G6 males suckled on "normal" mouse milk to ovine BLG. Each point is the data for serum from a single mouse.

a) Non-trangenic



b) Transgenic



NDAb = no detectable antibody

responses following each immunisation. As expected, transgenic mice were hyporesponsive to BLG as compared to non-transgenic littermates although significant differences in responses were only evident after the third immunisation in the case of male ($p<0.005$) and female ($p<0.005$) BLG-transgenics.

b) Female and male mice suckled on "normal" mouse milk (Fig 4.16 and 4.**18**).
Like above, BLG-transgenic and non-transgenic G6 male and female mice suckling on "normal" mouse milk produced anti-BLG IgG responses following each immunisation. As expected transgenic mice made a significantly reduced anti-BLG IgG response as compared to non-transgenic mice and significant differences in the responses were evident after each immunisation ($p<0.05$ (first) $p<0.005$ (second) and $p<0.01$ (third)) in the case of male G6 BLG-transgenics. No statistical analyses were done on the female data as only two G6 transgenic female mice were analysed.

c) Comparing the anti-BLG IgG responses of mice suckled on either BLG-containing or "normal" mouse milk.
To analyse whether suckling BLG-containing milk reduced systemic antibody responses to this protein the anti-BLG IgG responses of transgenic and non-transgenic mice suckled on BLG-containing milk were compared with the anti-BLG IgG responses of transgenic and non-transgenic mice suckled on "normal" mouse milk.
Suckling on BLG-containing milk did not reduce the anti-BLG IgG responses of non-transgenic and BLG-transgenic mice suggesting that backcrossing onto a CBA/Ca background did not result in mice capable of being orally tolerised to BLG through suckling BLG-containing milk.

4.4) Overall conclusions.

These experiments show that male and female mice heterozygous for the ovine BLG gene are hyporesponsive, at the

antibody level, to the product of this gene when exposed to ovine BLG and adjuvant administered parenterally. These mice were also hyporesponsive to the cross-reactive protein bovine BLG.

Reduced anti-BLG IgG responses could not be attributed to; 1) poor immunogenicity of this protein since non-transgenic and other groups of mice (see Chapter 2) made good secondary IgG responses to BLG, 2) to the exposure of these mice to BLG protein during pregnancy and lactation since transgenic offspring of CBA/Ca or non-transgenic mothers were also hyporesponsive to this protein.

These data are intriguing in the light of the absence of detectable transgene expression in female virgin and male mice. Even after multiple pregnancies and lactations female BLG-transgenic mice remain tolerant to BLG. Thus although expression of the BLG gene is specific to the lactating mammary gland the presence of the transgene in male and virgin female mice appears to cause this antibody tolerance.

The presence of the BLG-transgene or exposure to BLG during the neonatal period did not interfere with the ability to respond to an unrelated protein; male and female BLG-transgenics and non-transgenic mice made equivalent anti-OVA responses. Thus hyporesponsiveness in BLG-transgenic mice is specific to the BLG protein (both ovine and bovine) and does not reflect a generalised defect in the B cell compartment of these mice.

Although significantly reduced anti-BLG IgG responses were observed in female non-transgenic mice that had suckled on the BLG-containing milk (following a secondary immunisation) in one experiment pooling the anti-BLG IgG titres from female mice in all experiments, however eliminate the significance of this phenonomen. Male non-transgenic mice suckled on BLG-containing milk also showed no reductions in anti-BLG responses. These observations suggest that exposure to the BLG protein during the neonatal period does not induce oral tolerance. The possiblities that the lack of oral tolerance to BLG was MHC phenotype related or that bovine BLG presence in

the food, following weaning, interfered with tolerance were eliminated by the finding that no suckling-related tolerance to ovine BLG was found in mice on a CBA/Ca background or when mice were placed on a diet lacking bovine BLG.

CHAPTER 5

BLG-SPECIFIC T CELL RESPONSES IN BLG-TRANSGENIC MICE

As shown in Chapter 4, transgenic mice possing the ovine BLG transgene were hyporesponsive to ovine BLG; male and female transgenic mice produced a lower anti-ovine BLG specific antibody response as compared to non-transgenic littermates and CBA/Ca controls. This chapter details the results of experiments to test for T cell hyporesponsiveness to ovine and bovine BLG in BLG-transgenic mice both in vivo and in vitro.

5.1) T cell responses in heterozygous transgenic mice

Transgenic mice derived from mating line 45 homozygous BLG-transgenic males with CBA/Ca females were tested for T cell responsiveness to BLG in vivo or in vitro using either a footpad thickening assay or lymph node proliferation assay respectively.

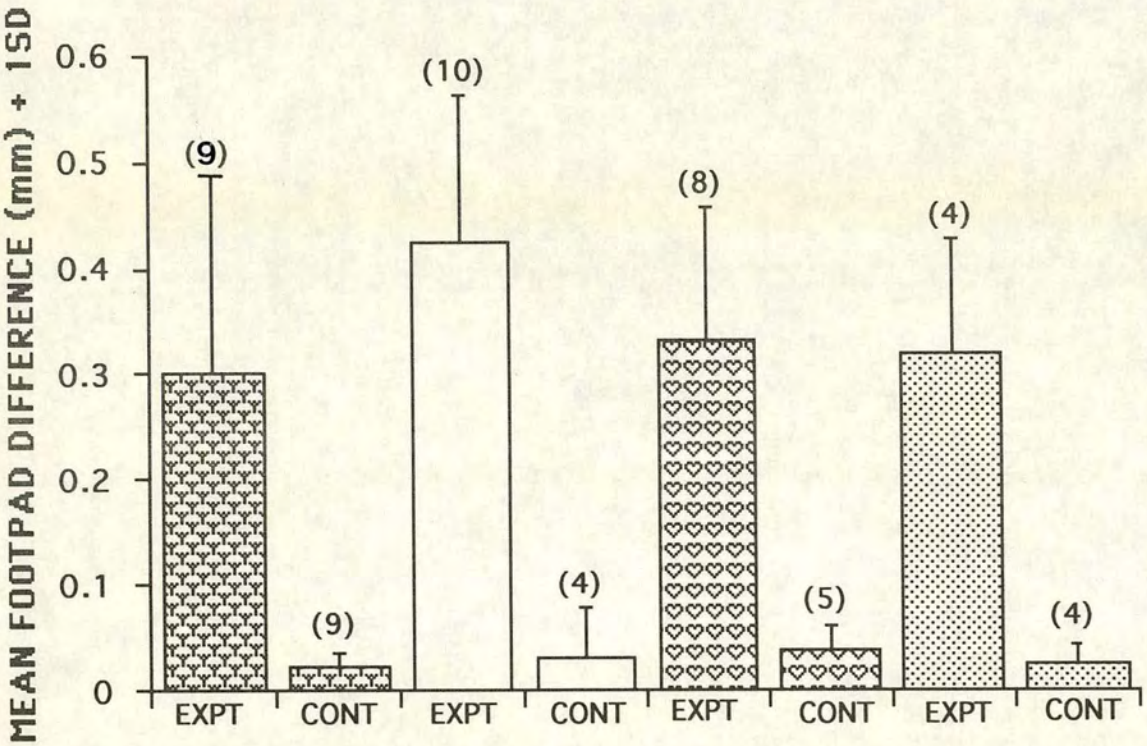
In the following sections the results described are from pooled experiments and unless otherwise stated agree with the results of individual experiments. Individual footpad data are shown in Appendices 11 to 14.

i) In vivo T cell responses to bovine BLG.

Heterozygous F1 BLG-transgenic male mice were immunised and challenged, with bovine BLG, for T cell responses as described (see Chapter 2). Control mice received a PBS secondary challenge. Figure 5.1 represents the pooled T cell data from 2 such experiments. Mice receiving PBS (the control mice) made significantly lower T cell responses ($p<0.001$) than those receiving heat aggregated (Hag) antigen.

Female F1 BLG-transgenic mice were also tested for T cell responses to bovine BLG (see Fig. 5.1) Once again a significant response to bovine BLG was observed ($p<0.01$) for mice given 2% Hag bovine BLG as compared to those challenged with PBS.

Fig. 5.1. T cell responses to bovine BLG.

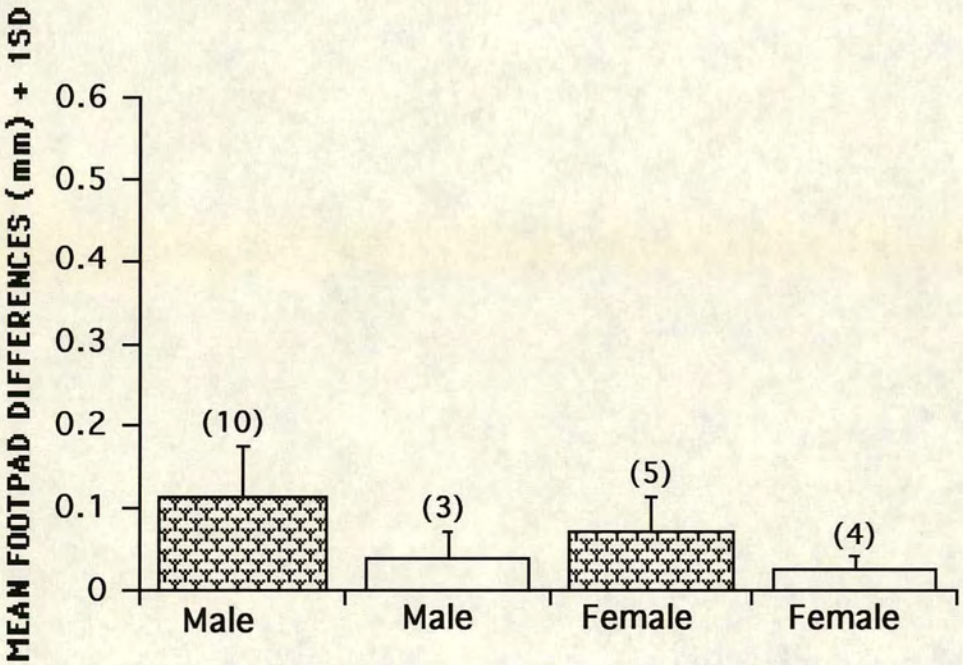


Mice were immunised with bovine BLG+FCA and 7 days later challenged with 2% Hag bovine BLG (EXPT) or PBS (CONT).
(nos)= number of mice tested

- CBA/Ca males
- F1 BLG-transgenic males
- CBA/Ca females
- F1 BLG-transgenic females

Error bars are +1SD

Fig. 5.2. T cell responses to ovine BLG.



F1 BLG-transgenic mice were immunised with ovine BLG and 7 days later challenged with 2% Hag ovine BLG or PBS.
(nos) = number of mice tested.
Error bars are + 1SD

2% Hag ovine BLG

PBS

Pooled T cell responses of F1 BLG-transgenic mice were compared with the pooled T cell responses of CBA/Ca mice (see Fig.5.1). Following a 2% Hag bovine BLG challenge both female and male F1 BLG-transgenic mice and CBA/Ca mice made comparable T cell responses to BLG. These data suggested that the presence of the BLG-transgene does not influence T cell responses to bovine BLG and the tolerance seen at the antibody level to bovine BLG is not evident at the T cell level.

ii) In vivo T cell response to ovine BLG.

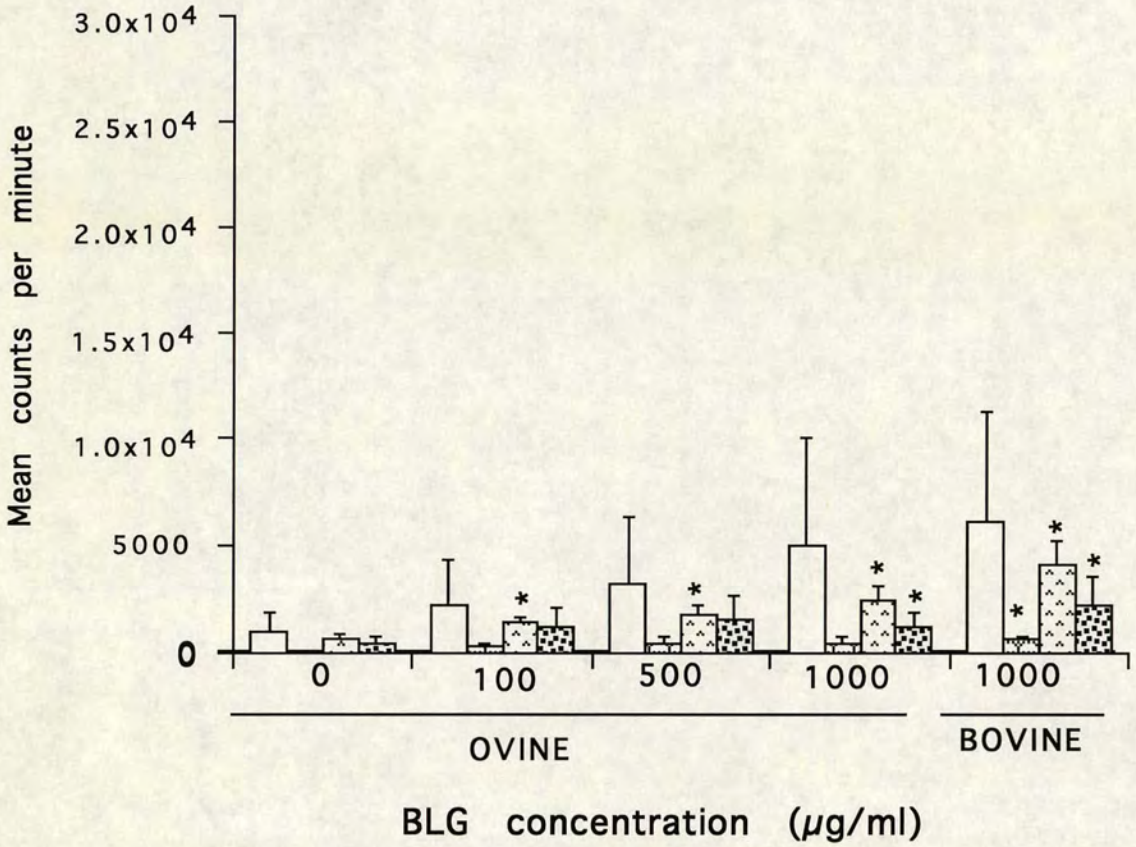
F1 BLG-transgenic male and female mice were immunised for T cell responses to ovine BLG and Fig. 5.2 represents data from one experiment. Male BLG-transgenic mice made small but significant secondary T cell responses to 2% Hag ovine BLG as compared to control mice challenged with PBS ($p<0.05$). This was not the case for female BLG-transgenic mice.

To test whether F1 males BLG-transgenic mice were hyporesponsive to ovine BLG, T cell responses of F1 BLG-transgenic mice were compared with the pooled T cell responses of male CBA/Ca mice (see Fig.3.6a, Chapter 3). Following a 2% Hag ovine BLG challenge male F1 BLG-transgenic mice and CBA/Ca mice made comparable T cell responses to BLG. These data suggested that the presence of the BLG-transgene does not influence T cell responses to ovine BLG for male BLG-transgenic mice although the responses were small.

iii) In vitro T cell responses

Two experiments were set up to look at the T cell responses to ovine BLG and the cross-reactivity of ovine BLG primed T cells to bovine BLG in vitro. Mice were immunised in the footpad with a mixture of antigen plus an Alum or FCA adjuvant and in vitro T cell assays were set up as described in the Materials and Methods (see Chapter 2). Figures 5.3 and 5.4a show the in vitro T cell responses following either an antigen plus Alum or FCA immunisation, respectively. Fig. 5.4b is the Con A control data.

Fig. 5.3. In vitro T cell responses to ovine and bovine BLG following immunisation with ovine BLG plus Alum

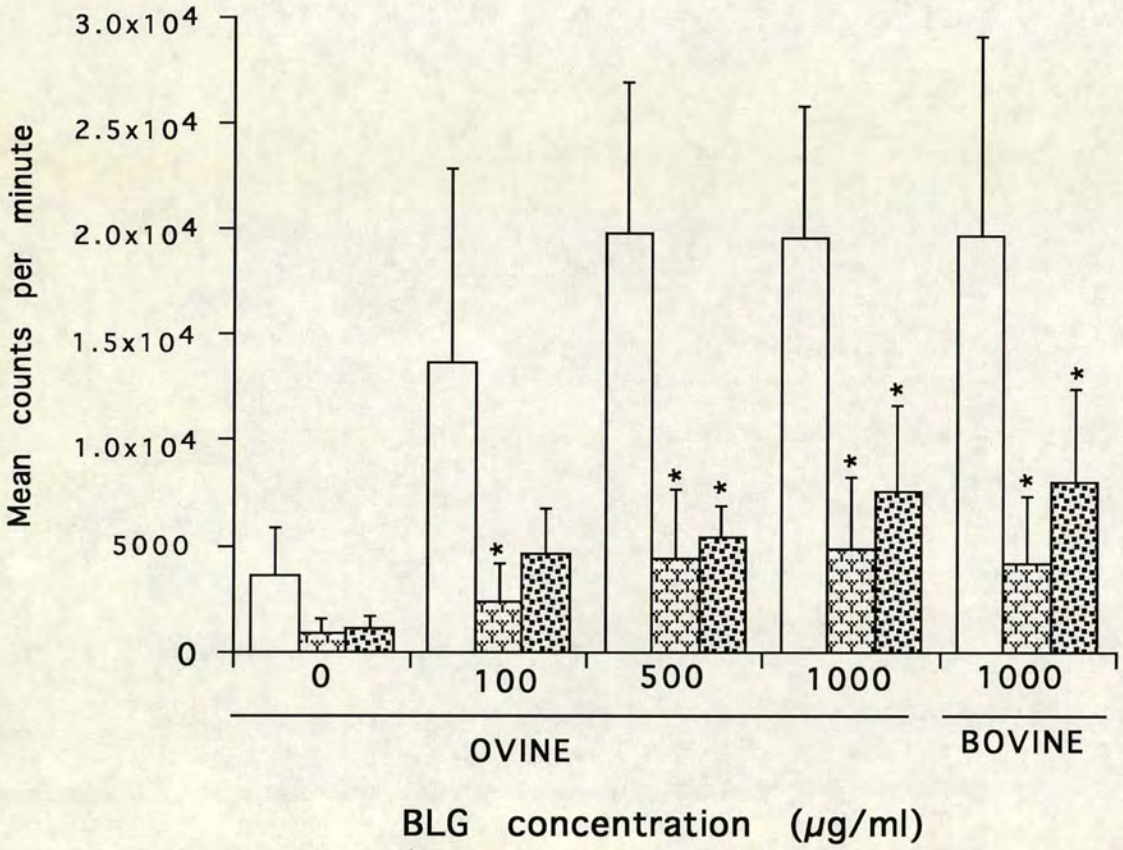


Mice were immunised with ovine BLG + Alum and 7 days later popliteal lymph nodes were removed. For each node a cell suspension of $2.5x10^6$ cells per ml was made. 100μls of this suspension was cultured for 3 days with either 100, 500, 1000μgs/ml of ovine BLG or 1000μgs/ml of bovine BLG. Proliferation of cells was measured using ^{3}H-tritium incorporation and is given as mean counts per minute.

□ CBA/Ca males (n=5)
▨ BLG F1 males (n=4)
▫ CBA/Ca females (n=4)
▩ BLG F1 females (n=5)

* = responses to BLG is significantly greater than the control ($p<0.05$)

Fig. 5.4a. In vitro T cell responses to ovine and bovine BLG following immunisation with ovine BLG plus FCA



Mice were immunised with ovine BLG + FCA and 7 days later popliteal lymph nodes were removed. For each node a cell suspension of $2.5x10^6$ cells per ml was made. 100μls of this suspension was cultured for 3 days with either 100, 500, 1000μgs/ml of ovine BLG or 1000μgs/ml of bovine BLG. Proliferation of cells was measured using ^{3}H-tritium incorporation and is given as mean counts per minute.

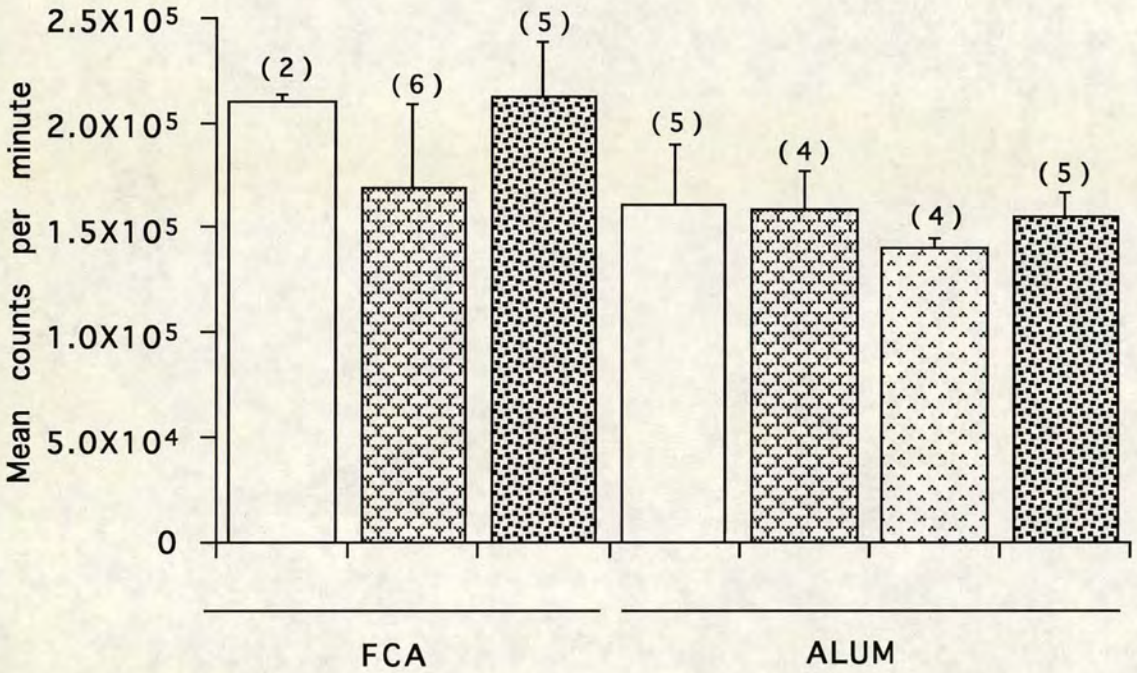
□ CBA/Ca males (n=2)

▨ BLG F1 Males (n=6)

▨ BLG F1 Females (n=5)

* = responses to BLG is significantly greater than the control ($p<0.05$)

Fig. 5.4b. In vitro T cell responses to Con A stimulation following immunisation with ovine BLG with either FCA or Alum.



Mice were immunised with ovine BLG + FCA or Alum and 7 days later the popliteal lymph nodes were removed and a $2.5x10^6$ cell suspension was made. Cells were stimulated with 4μg/ml of Con A for 3 days. Proliferation was measured using ^{3}H-tritium incorporation.

- CBA/Ca males
- CBA/Ca females
- BLG F1 males
- BLG F1 females

(nos)= number of mice tested

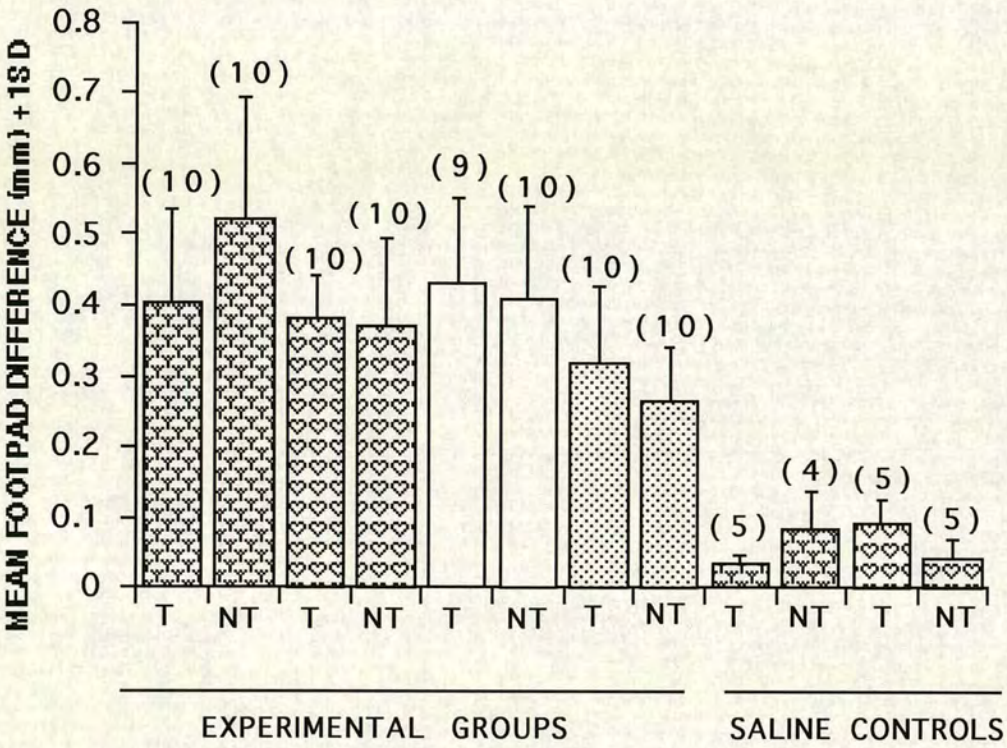
The mean stimulation indices of lymph node cells stimulated with either 10, 50, 100μgs of ovine BLG or 100μgs of bovine BLG from female BLG-transgenic and CBA/Ca mice immunised with ovine BLG plus Alum, were compared using a 2 sample T-test. BLG-transgenic mice made equivalent in vitro T cell responses to both 10 and 100μgs of ovine BLG to those of CBA/Ca mice. Similarly ovine BLG primed lymph node cells from both CBA/Ca and BLG-transgenic female mice made equivalent responses to bovine BLG. Stimulation in vitro with 50μgs of ovine BLG resulted in transgenic cells making a significantly greater response to this antigen concentrations than lymph node cells from CBA/Ca mice, ($p<0.01$).

Ovine BLG primed lymph node cells from male BLG-transgenic mice also responded to the three concentrations of ovine BLG to the same extent as CBA/Ca mouse cells. Differences between these two groups of mice were only seen after stimulation with bovine BLG, F1 BLG-transgenic mice made significantly reduced response to this protein ($p<0.05$) compared to CBA/Ca mice.

Lymph node cells from ovine BLG and FCA primed BLG-transgenic and CBA/Ca male mice were stimulated in vitro with ovine and bovine BLG. The mean stimulation indices for these groups are shown in **Fig 5-4**. Lymph node cells from BLG primed F1 BLG-transgenic and CBA/Ca mice proliferated to each ovine BLG concentration and bovine BLG similarly. Although no in vitro T cell data to ovine BLG were available for CBA/Ca female mice the mean stimulation indices of female F1 BLG-transgenic mice lymph node cells stimulated in vitro with ovine BLG were compared with the male transgenic response. Comparing the stimulation indices of female with male transgenic mice suggested that both sexes made equivalent responses, although female lymph node cells responded better to a 10μgs stimulation of ovine BLG ($p<0.01$).

Taken together with the in vivo data, it would appear that no T cell tolerance following injection of either ovine and bovine BLG with Alum or FCA was observed in F1 BLG-transgenic.

Fig 5.5. T cell responses to bovine BLG



BLG-transgenic (T) and non-transgenic (NT) mice were immunised with bovine BLG + FCA and 7 days later mice were challenged with 2% Hag bovine BLG (EXPT) or PBS (CONT).
(nos)= number of mice tested

- Male mice suckled on BLG-containing milk (S)
- Female mice suckled on BLG-containing milk(S)
- Male mice suckled on"normal" mouse milk (NS)
- Female mice suckled on "normal" mouse milk (NS)

Error bars are + 1SD

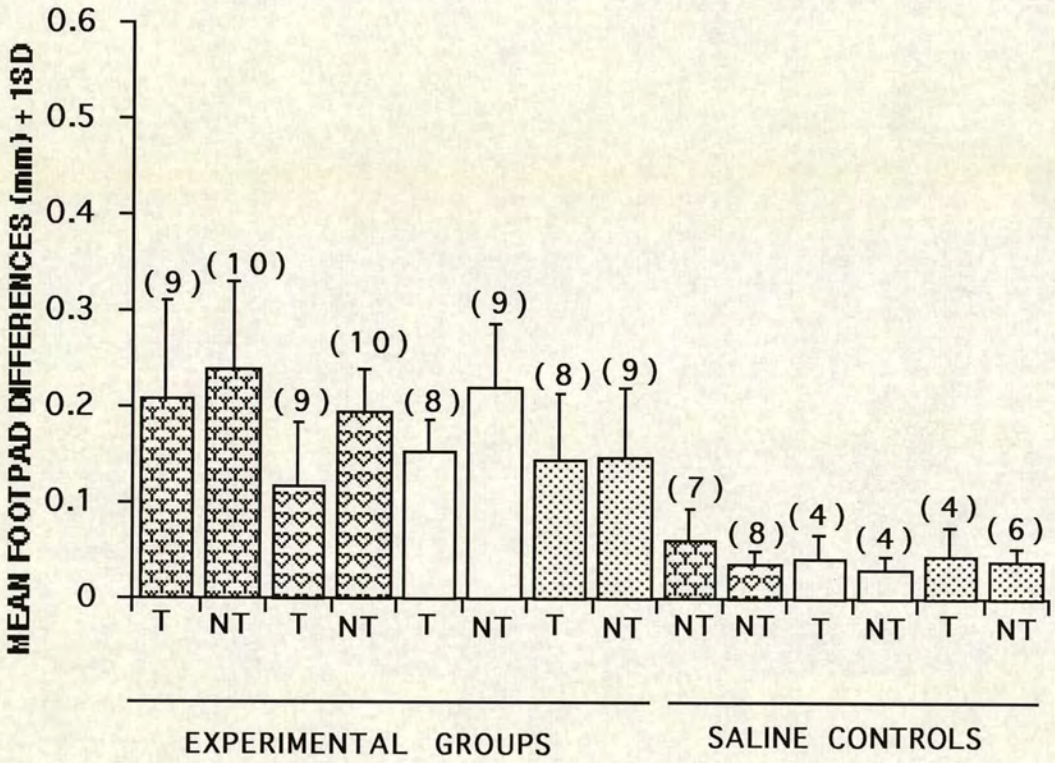
5.2) T cell responses in BLG-suckled and non-suckled transgenic littermates.

BLG-transgenic and non-transgenic mice derived from mating F1 heterozygous BLG-transgenic mice with CBA/Ca partners (see Fig. 4.7, Chapter 4) in such a way that offspring were either exposed to BLG via milk produced from lactating transgenic mother's or suckled on "normal" CBA/Ca mouse milk were tested for T cell responses in vivo. These crosses were set up to investigate whether suckling BLG-containing milk would influence the development of T cell responses to BLG and to compare the T cell responses of BLG-transgenic mice with those of their non-transgenic littermates.
In the sections below the T cell data are pooled from more than one experiment

i) In vivo T cell responses to bovine BLG.

3 month old mice were identified as BLG-transgenic or non-transgenic mice and were immunised for T cell analysis as described (see Chapter 2). The results of two such experiments are described in Fig. 5.**5**. All groups of mice challenged with 2% Hag bovine BLG made significantly greater responses than control mice challenged with saline ($p<0.001$ for all groups). No significant differences were observed between experimental groups; BLG-transgenic and non-transgenic mice made equivalent T cell responses to bovine BLG.
The T cell responses of mice suckled on BLG-containing milk were equivalent to those of mice suckled on "normal" mouse milk with one exception. Non-transgenic female suckled on BLG-containing milk made significantly greater T cell responses than non-transgenic mice suckled on "normal" mouse milk ($p<0.05$). The former observation was not found in either of the individual experiments.
In conclusion, BLG-transgenic mice were not tolerant to bovine BLG at the T cell level in vivo and suckling on BLG-containing milk did not influences these responses.

Fig 5.6. T cell responses to ovine BLG.

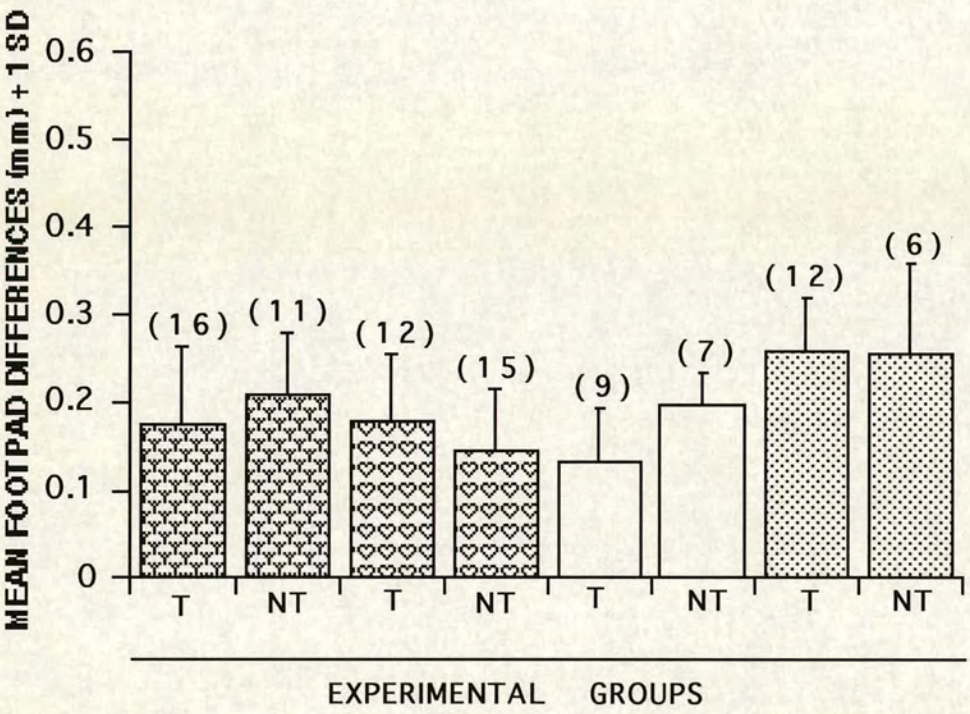


BLG-transgenic (T) and non-transgenic (NT) mice were immunised with ovine BLG+FCA and 7 days later challenged with 2% Hag ovine BLG (EXPT) or PBS (CONT). (nos)= number of mice tested

- Male mice suckled on BLG-containing milk (S)
- Female mice suckled on BLG-containing milk (S)
- Male mice suckled on "normal" mouse milk (NS)
- Female mice suckled on "normal" mouse milk (NS)

Error bars are + 1SD

Fig 5.7. T cell responses to ovine BLG.



BLG-Transgenic (T) and non-transgenic (NT) mice, maintained on a diet lacking whey protein, were immunised with ovine BLG+FCA and 7 days later challenged with 2% Hag ovine BLG.
(nos)= number of mice tested.

Male mice suckled on BLG-containing milk (S)
Female mice suckled on BLG-containing milk (S)
Male mice suckled on "normal" mouse milk (NS)
Female mice suckled on "normal" mouse milk (NS)

Error bars are +1SD

ii) In vivo T cell responses to ovine BLG.

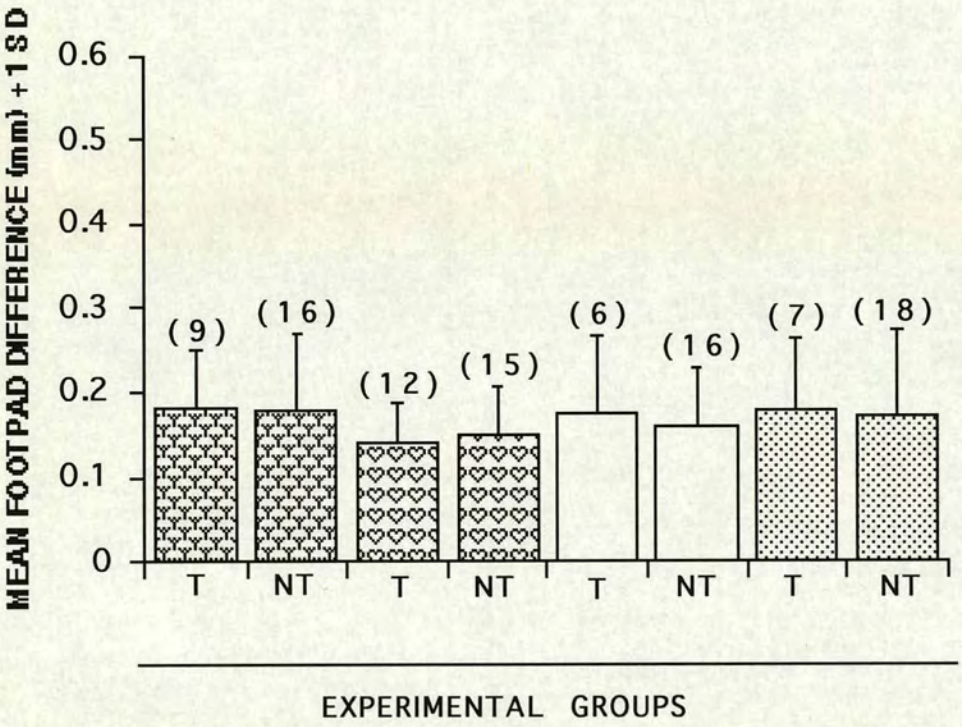
Groups of BLG-transgenic and non-transgenic littermate mice either suckled on BLG-containing or "normal" mouse milk were tested for T cell responses to ovine BLG. Two experiments were performed see Fig. 5.6. Although the overall T cell responses to ovine BLG were less than for bovine BLG the results were similar. There were significant increases in all experimental groups challenged with 2% Hag ovine BLG compared to controls ($p<0.01$). Like the bovine BLG data, male transgenic mice suckled on BLG-containing milk as well as female transgenic mice suckled on "normal" mouse milk made equivalent T cell responses to 2% Hag BLG as compared to their non-transgenic equivalents. In contrast, female transgenic mice suckled on BLG-containing milk made significantly lower T cell responses to ovine BLG ($P<0.01$) than equivalent non-transgenic littermates. The same was observed for male transgenic mice suckled on "normal" mouse milk ($P<0.05$). However when the individual experiments were analysed separately, the above differences between transgenic and non-transgenic mice were only found in one experiment.

Suckling BLG-containing milk did not result in reduced T cell responses.

This experiment was repeated on mice from the above crosses who following suckling were placed on a diet lacking whey protein. At three months of age these mice were immunised and challenged with ovine BLG. No saline controls were included. This experiment was performed twice, the pooled T cell responses are shown in Fig. 5.7. No difference was found between transgenic and non-transgenic mice with one exception; male transgenic mice suckled on "normal" mouse milk made significantly reduced T cell responses compared to their non-transgenic littermates ($p<0.05$). It should be noted that the T cell responses of male BLG-non-suckled mice were from one experiment.

Suckling BLG-containing milk did not result in reduced T cell responses to ovine BLG with one exception; transgenic females suckled on BLG-containing milk had significantly reduced

Fig 5.8. T cell responses to ovine BLG

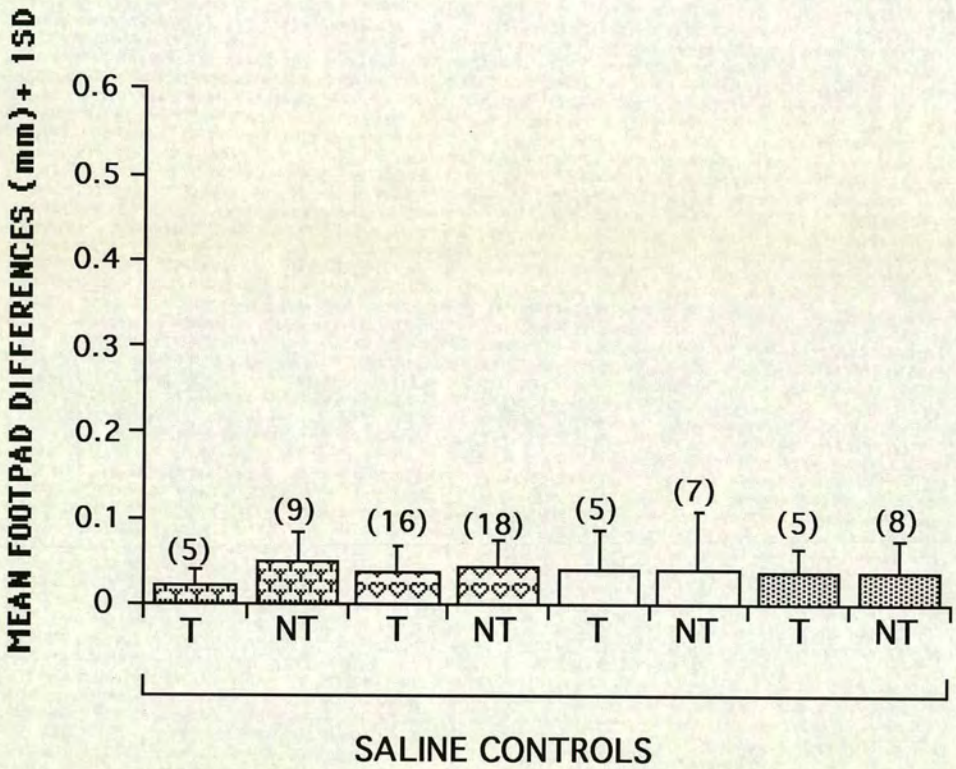


G7 backcrossed BLG-transgenic (T) and non-transgenic (NT) mice were immunised with ovine BLG+FCA and 7 days later challenged with 2% Hag ovine BLG.
(nos) = number of mice tested.

Male mice suckled on BLG-containing milk (S)
Female mice suckled on BLG-containing milk (S)
Male mice suckled on "normal" mouse milk (NS)
Female mice suckled on "normal" mouse milk (NS)

Error bars are + 1SD

Fig. 5.9. T cell responses to saline



G7 backcrossed BLG-transgenic (T) and non-transgenic (NT) mice were immunised with ovine BLG+FCA and 7 days later challenged with PBS.
(nos)= number of mice tested.

Male mice suckled on BLG-containing milk (S)

Female mice suckled on BLG-containing milk (S)

Male mice suckled on "normal" mouse milk(NS)

Female mice suckled on "normal" mouse milk (NS)

Error bars are + 1SD

responses ($p<0.01$) as compared to transgenic females suckled on "normal" mouse milk. When experiments were analysed separately this observation was found in only one experiment. It was also observed in this experiment that non-transgenic females suckled on BLG-containing milk made significantly reduced T cell responses ($p<0.01$) to ovine BLG as compared to non-transgenic females suckled on "normal" mouse milk. Only two mice were assayed in this group.

5.3) T cell responses in BLG-suckled and non-suckled G7 transgenic littermates.

The data indicate that in most cases BLG-transgenic and non-transgenic mice make comparable T cell responses following either a ovine or bovine BLG challenge suggesting that no T cell tolerance to BLG had occurred in BLG-transgenic mice. One hypothesis to explain this result was that any differences at the T cell level were difficult to detect due to the heterogeneous MHC composition of the offspring from the original crosses. The effects of this segregating background were reduced by backcrossing onto a CBA/Ca background. Heterozygous transgenic and non-transgenic backcrossed generation 6 (G6) mice were mated as described in Fig.4.7 (see Chapter 4). At three months of age G7 BLG-transgenic or non-transgenic animals were immunised and challenged with 2% Hag ovine BLG as previously described. This experiment was repeated twice and Fig. 5.**8** shows the T cell responses from the pool of these experiments and Fig. 5.**9** the T cell responses of G7 mice challenged with saline.

i) In vivo responses to ovine BLG.

Backcrossing onto a CBA/Ca background resulted in both transgenic and non-transgenic mice making significant T cell responses ($p<0.01$) to a 2% Hag ovine BLG challenge as compared to saline challenged control mice. The T cell responses of the former experimental groups were equivalent. Suckling BLG-containing milk did not reduce the T cell response to this protein. In one experiment however (data not

shown) female non-transgenic mice suckled on BLG-containing milk made significantly lower T cell responses to ovine BLG than non-transgenic mice not exposed to this protein ($p<0.001$). This effect disappeared after data were pooled.

5.4) T cell summary

In some in vivo experiments BLG-transgenic mice had reduced T cell responses to ovine BLG as compared to their non-transgenic littermates, however this was not the case in the majority of experiments. Even when mice were primed with ovine BLG and challenged with bovine BLG, which elicits better secondary footpad thickening, evidence for reduced T cell responses in BLG-transgenic mice was not found (data not shown, see Appendix 15). In vitro assays also indicated that no difference in the ability to respond at the T cell level was evident between BLG-transgenic and CBA/Ca mice. Taken together with the above information and the fact that transgenic and non-transgenic mice produce equivalent T cell responses to bovine BLG it can be concluded that use of the footpad thickening in vivo assay and in vitro lymph node proliferation assays provided little evidence for hyporesponsiveness at the T cell level in BLG-transgenic mice.

CHAPTER 6

BONE MARROW STUDIES

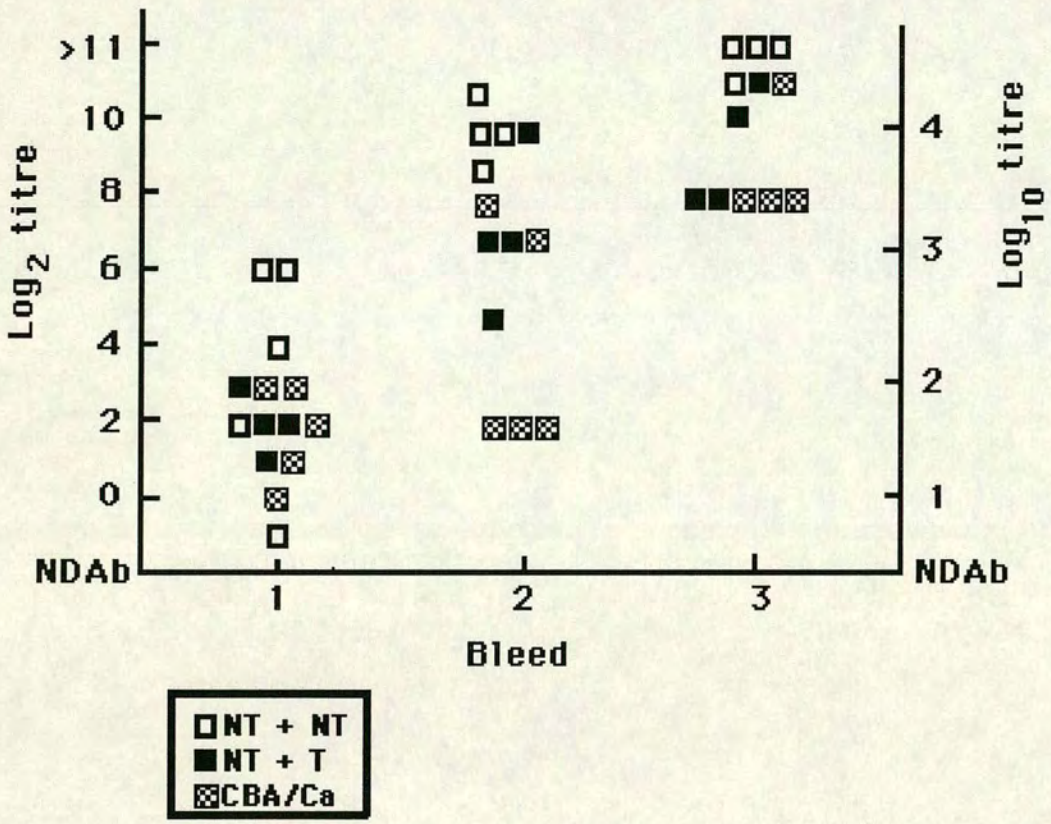
Bone marrow chimaeras have been used extensively to dissect B and T cell tolerance mechanisms and are used in this chapter to determine whether hyporesponsiveness is an intrinsic property of transgenic lymphoid cells or due to expression of the transgene in bone marrow, thymus or other sites in the periphery. Goodnow et al (1988) have shown that both the HEL transgenic mice and double transgenic (Dbl-tg) mice expressing both HEL and anti-HEL antibody transgenes are tolerant at the antibody level to HEL. Using bone marrow chimaeras Adams et al (1990) demonstrated in this case that hyporesponsiveness was an intrinsic property of the lymphoid cells themselves. Tolerance to HEL was induced in non-transgenic mice reconstituted with bone marrow from a Dbl-tg mouse even when primed T cells were also transferred. In contrast, bone marrow experiments performed by Nemazee et al (1989) in another transgenic system showed that tolerance was not an intrinsic property of the transgenic bone marrow derived B cells. Bone marrow, depleted of T cells, from transgenic mice with B cells specific for H-2K^{k} when transferred into an H-2K^{d} mouse resulted in immunological tolerance to H-2K^{k}. This was not the case when transgenic bone marrow was transferred into H-2D^{d} mice.

Bone marrow chimaeras have also been used to investigate T cell tolerance within the thymus. These experiments highlighted the importance of bone marrow derived cells, located within the thymus, in the removal of self-reactive T cells and also provided evidence that thymic epithelial cells possessing self antigens can be responsible for T cell tolerance although through a process of anergy rather than deletion. All these experiments are described in detail in the introduction (see Chapter 1).

Fig. 6.1

Antibody responses of male bone marrow chimaeric mice and unirradiated CBA/Ca mice to ovine BLG. Each point is the data for serum from a single mouse.

a) Non-transgenic (NT) recipients of either NT or BLG-transgenic (T) bone marrow.

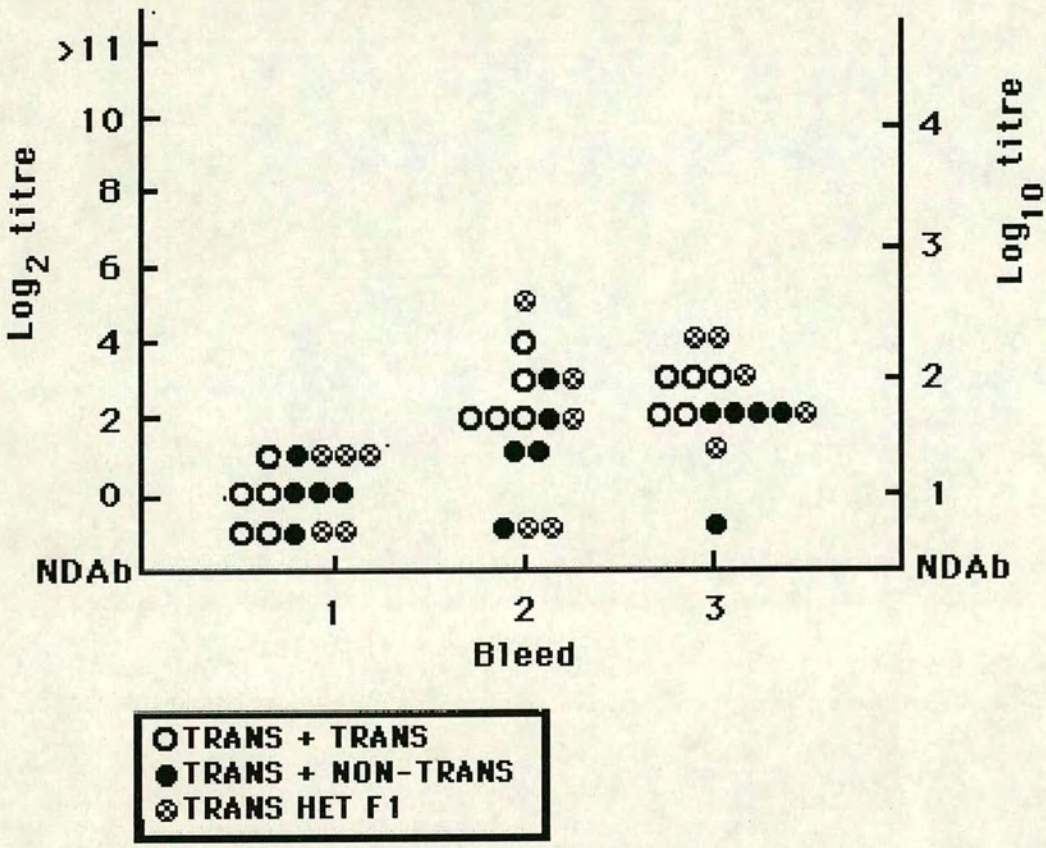


NDAb= no detectable antibody.

Fig. 6.1 cont

Antibody responses of male bone marrow chimaeric mice and unirradiated F1 BLG-transgenic mice to ovine BLG. Each point is the data for serum from a single mouse.

b) Transgenic recipients (T) of either NT or T bone marrow.



NDAb= no detectable antibody.

As described in Chapter 4, BLG-transgenic male and female mice are immunologically hyporesponsive to both ovine and bovine BLG at the antibody level. To assess whether this is an intrinsic property of the immune cells themselves, in that they are unable to respond due to the effects of the transgene on T and B cells development, or due to an environmental effect, expression of the transgene within the animal resulting in silencing of self-reactive T or B cell clones, bone marrow chimaeras were established (see Materials and Methods, Chapter 2). T cells were not removed from the bone marrow preparations.

6.1) Male bone marrow chimaeras

In the first set of these experiments offspring from F1 BLG-transgenic X CBA/Ca, and the reciprocal crosses were used as both bone marrow donors and recipients, transgenic and non-transgenic mice having been identified by PCR.

Two experiments of this kind were set up. In the first the mice were 9 months of age; in the second the mice were younger, 3-4 months old. Following 10.5Gy of gamma irradiation, recipient mice were given either 10^7 BLG-transgenic or non-transgenic littermate bone marrow intravenously (see Materials and Methods, Chapter 2). 3 months later they were immunised and antibody titres analysed as shown in Figs 6.1 and 6.2.

In experiment one (Fig. 6.1), 6 groups of mice were set up including 4 control groups. Two of the control groups of mice received autologous bone marrow and were designated T+T (transgenic recipients given transgenic bone marrow) and NT+NT (non-transgenic mice given non-transgenic bone marrow). The other control groups (CBA/Ca and F1 BLG-transgenic mice) did not receive any bone marrow and no irradiation. Experimental groups received heterologous bone marrow and were designated T+NT (transgenic mice reconstituted with non-transgenic bone marrow) and NT+T (non-transgenic mice reconstituted with transgenic bone marrow).

Three months following bone marrow reconstitution mice were immunised with ovine BLG and antibody responses analysed as described. T+T control mice made low primary anti-ovine BLG IgG antibody titres following the first immunisation (ranging from no detectable antibody to 0) whilst NT+NT control mice made detectable levels of antibody (one mouse had no detectable antibodies; in the other mice titres ranged from 2 to greater than 5). Following a secondary immunisation there was a slight but significant increase in the secondary titres for the T+T (range 2 to 4, $p<0.05$) group and a much larger increase for the NT+NT group (range 9 to 11, $p<0.01$). A further increase in anti-ovine BLG IgG titres occurred following a third immunisation for the NT+NT group (titres were >11, $p<0.01$). Comparing these two groups indicated a significantly reduced antibody response at both the primary and secondary antibody level for the T+T groups, hence confirming that these mice are hyporesponsive to BLG. This was further confirmed by the controls in this experiment; unirradiated F1 BLG-transgenic control mice were hyporesponsive compared to unirradiated CBA/Ca following each immunistion with ovine BLG.

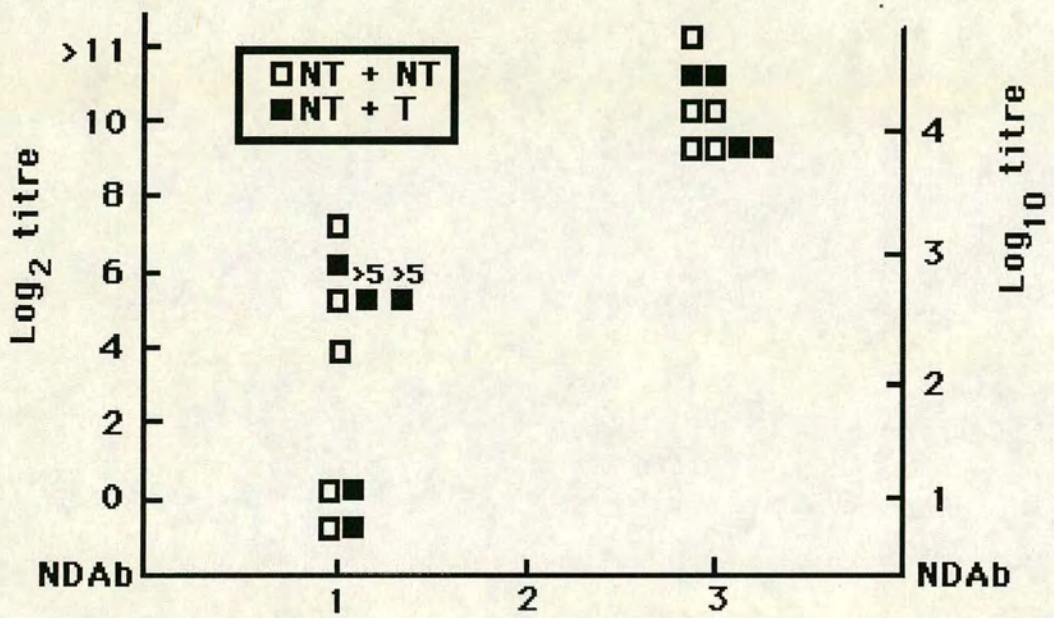
Reconstituting ovine BLG-hyporesponsive BLG-transgenic mice with bone marrow from mice already shown to be responsive to this antigen (T+NT group) did not result in mice capable of producing high primary and secondary IgG titres to ovine BLG. Comparing the primary and secondary anti-ovine BLG IgG titres of T+T and T+NT groups of mice revealed that each group of mice made equivalent responses after each immunisation. The responses of T+NT mice and F1 BLG-transgenic mice were also comparable.

In contrast the injection of bone marrow from mice shown to be tolerent to BLG into mice that were capable of responding (NT+T group) demonstrated that in a non-transgenic environment bone marrow cells from BLG-transgenic mice developed normal antibody responses to ovine BLG following exposure to this antigen. The NT+T mice made comparable

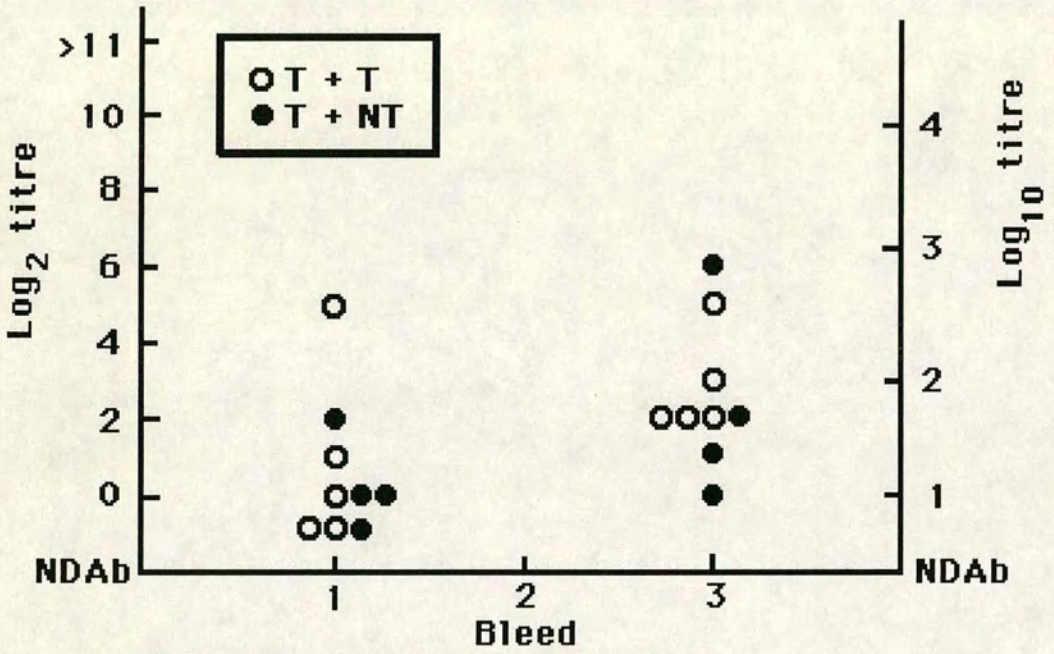
Fig. 6.2

Antibody responses of male bone marrow chimaeric mice to ovine BLG (repeat EXPT) Each point is the data for serum from a single mouse

a) Non-transgenic recipient mice



b) Transgenic recipient mice

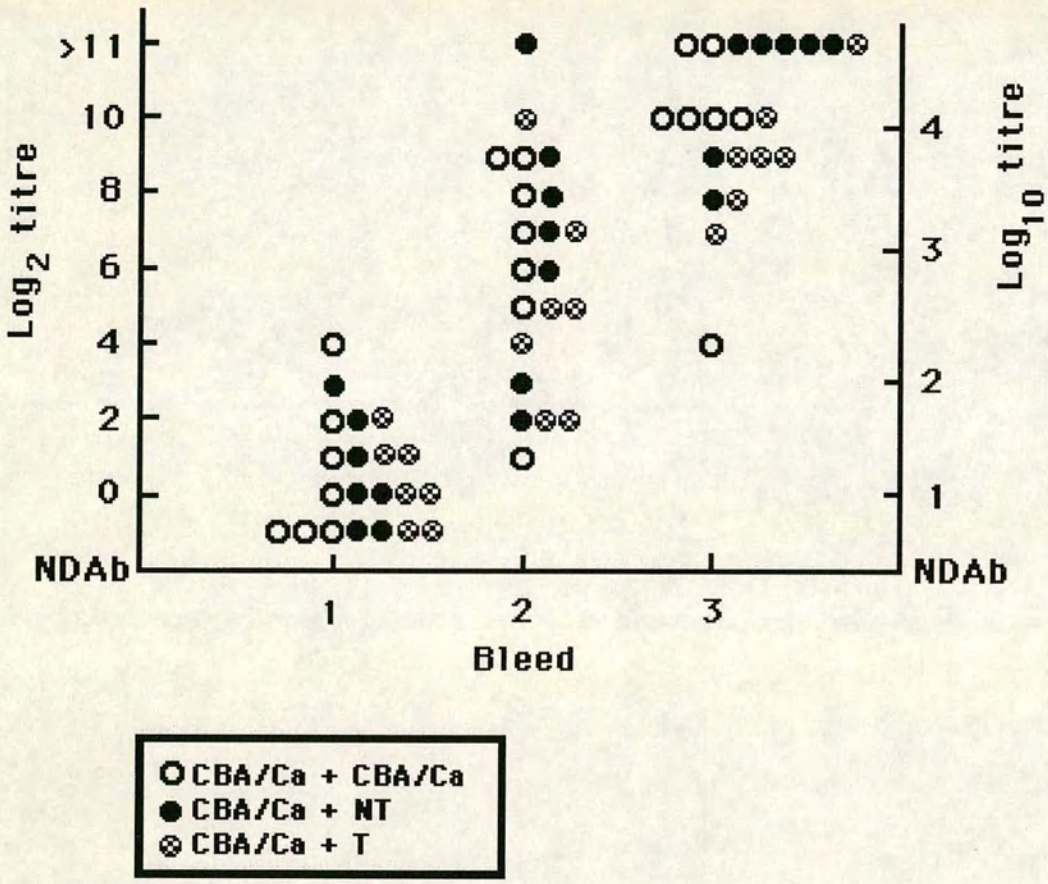


NDAb= no detectable antibody

Fig. 6.3

Antibody responses of male bone marrow chimaeric mice to ovine BLG. Each point is the data for serum from a single mouse.

CBA/Ca recipients reconstituted with CBA/Ca, non-transgenic (NT) and transgenic (T) bone marrow.



NDAb= no detectable antibody.

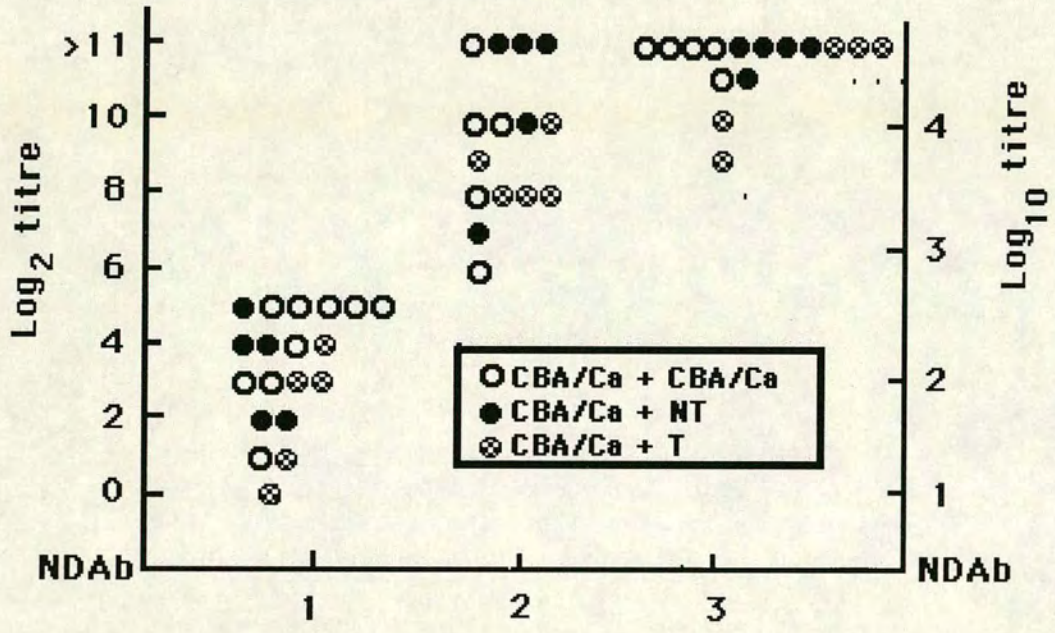
primary and secondary anti-BLG IgG titres to those of NT+NT and CBA/Ca mice.
The second experiment confirmed the observations above (Fig. 6.2). In this experiment only comparisons of the primary (1st bleed) and the tertiary responses (3rd bleed) were made. No significant increase in anti-ovine BLG IgG titres occurred in the T+T and T+NT groups. However NT+NT and NT+T mice made significant secondary antibody response to ovine BLG ($p<0.01$).

Since the original transgene founders were on a C57BL/6 X CBA/Ca heterozygous background it was possible that some histocompatibility differences between donor marrow and recipient occurred. Although at the gross level there was no evidence for graft vs host disease, the experiment above was repeated after the transgene had been backcrossed onto a CBA/Ca background. Bone marrow transplantation experiments were performed on backcross generations 5 and 6 (G5 and G6 respectively) mice.
In the first experiment (Fig. 6.3), irradiated 3 month old CBA/Ca mice were reconstituted with G5 transgenic (C+T) or non-transgenic (C+NT) bone marrow. Contol mice were given CBA/Ca bone marrow (C+C). Mice were immunised for responses to ovine BLG as described in the Materials and Methods, (see Chapter 2) and the titres following primary and secondary immunisation are shown in Fig. 6.3.
A first immunisation with ovine BLG resulted in low levels of anti-ovine IgG titres (range for C+T was from no detectable antibody to 2, for C+NT the titre range was from no detectable antibody to 3 and from no detectable antibody to 4 for C+C). A secondary immunisation induced a significant increase in anti-IgG titres for each group (C+T $p<0.01$, C+NT $p<0.01$ and C+C $p<0.01$). This also occurred following a third challenge (C+T $p<0.01$, C+NT $p<0.01$ and C+C $p<0.01$).
Comparisons of the antibody responses of each group indicated that the three groups of mice made similar primary and secondary responses. Thus reconstituting mice capable of producing antibodies to BLG (CBA/Ca) with bone marrow from

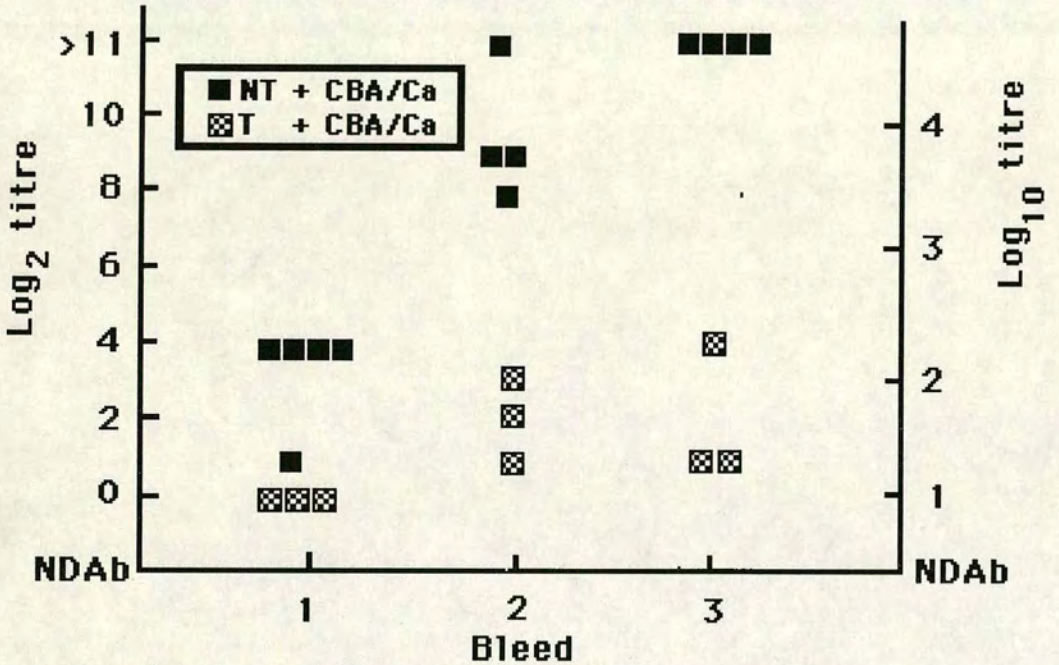
Fig. 6.4.

Antibody responses of male bone marrow chimaeric mice to ovine BLG (repeat EXPT)
Each point is the data for serum from a single mouse.

a) CBA/Ca recipients



b) Non-transgenic and transgenic recipients



NDAb= no detectable antibody.

transgenic mice did not induce hyporesponsiveness to BLG; anti-ovine BLG IgG responses of C+T groups were equivalent to those of CBA/Ca mice reconstituted with CBA/Ca or non-transgenic bone marrow.
This experiment was repeated and the same observations were noted, see Fig. 6.4. In this experiment however transgenic and non-transgenic G6 mice were reconstituted with CBA/Ca bone marrow (T+C and NT+C, respectively). The T+C mice did not make significant secondary antibody response to ovine BLG whereas the opposite was seen for non-transgenic G6 littermates reconstituted with CBA/Ca (NT+C, $p<0.05$). Non-transgenic mice receiving CBA/Ca bone marrow thus made a greater secondary response to BLG than transgenic mice receiving CBA/Ca bone marrow ($p<0.05$). T+C mice also made a significantly reduced secondary antibody response in comparison to C+T mice ($p<0.01$).

In conclusion, male BLG-transgenic mice remain hyporesponsive to ovine BLG despite being reconstituted with bone marrow from an animal already shown to be capable of mounting an immune response. However when bone marrow from animals (BLG-transgenic mice) shown to be non-responsive to BLG was used to reconstitute mice not possessing the gene the hyporesponsiveness was abrogated. Hence, bone marrow derived cells from a transgenic mouse have the capability to respond to ovine BLG. Environmental effects such as the expression of the transgene either in bone marrow stroma or in the periphery lead to the silencing or deletion of cells capable of responding to this protein.

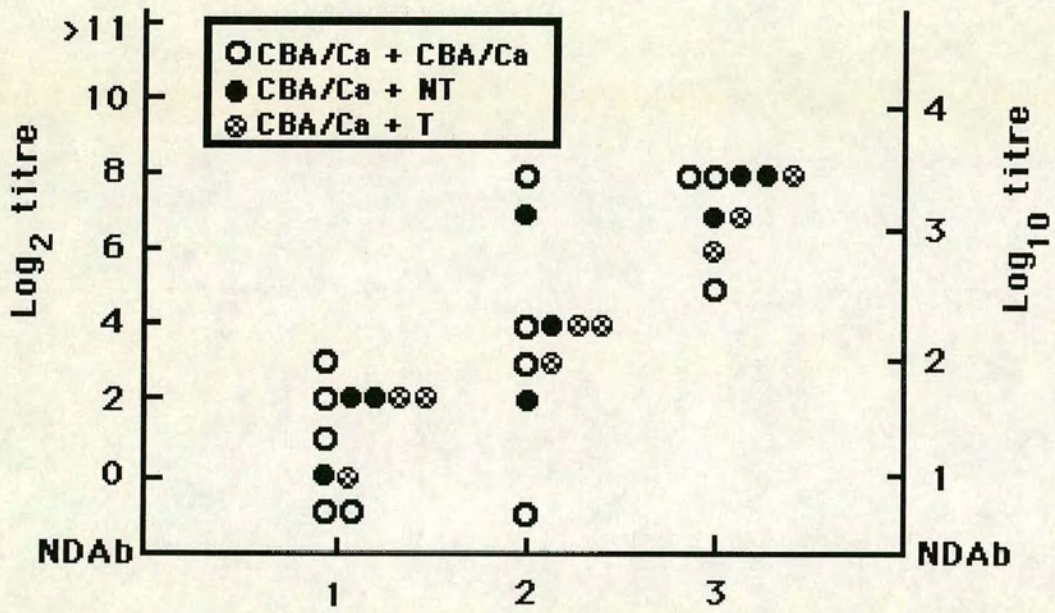
6.2) Female bone marrow chimaeras

Bone marrow transfer experiments were also carried out using female donor and recipient mice. In these experiments recipient and donor mice were from a G5 backcrossed population. Again control mice were given autologous bone marrow and these included C+C, NT+NT and T+T groups, whilst experimental mice were given allogeneic bone marrow. The

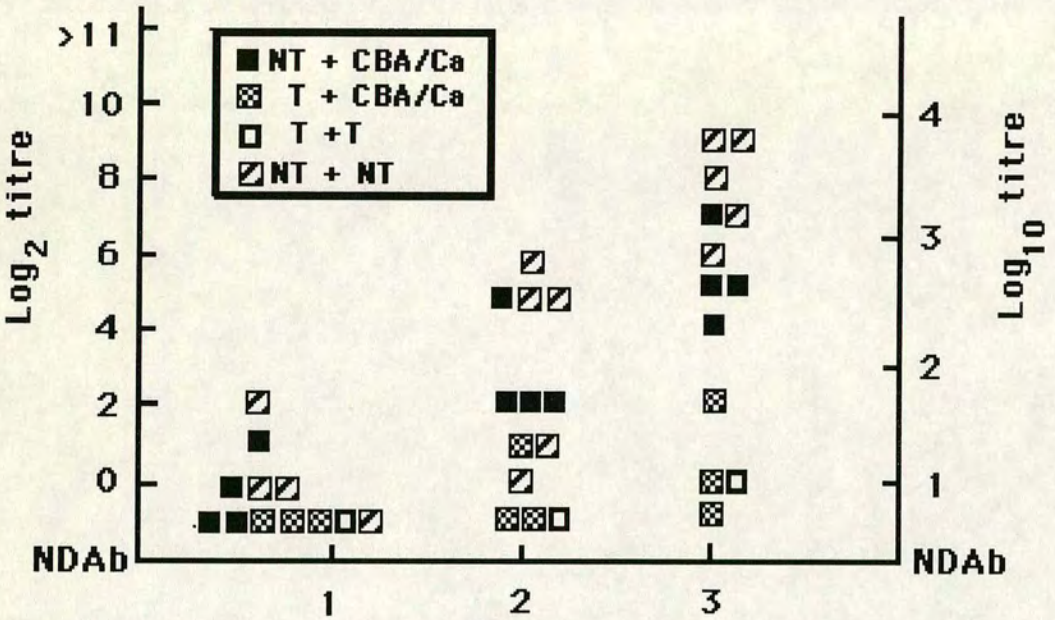
Fig. 6.5

Antibody responses of female bone marrow chimaeric mice to ovine BLG. Each point is the data for serum from a single mouse

a) CBA/Ca recipients



b) Non-transgenic and transgenic recipients



NDAb= no detectable antibody

experimental groups were T+C and NT+C (transgenic and non-transgenic mice reconstituted with CBA/Ca bone marrow), C+T and C+NT (CBA/Ca mice reconstituted with either transgenic and non-transgenic bone marrow respectively) see Fig. 6.5.
The results are essentially similar to those described for male animals and confirm the necessity for non-lymphoid expression of the transgene to induce hyporesponsiveness.

6.3) Conclusion

The genotype of the recipient dictated the immune status of the animal for both male and female mice; transgenic mice given CBA/Ca or NT bone marrow remained significantly hyporesponsive to ovine BLG. In contrast CBA/Ca or NT mice given transgenic bone marrow remained as responsive to BLG immunisation as the non-irradiated controls. Hyporesponsiveness to BLG is thus not transferable with transgenic bone marrow. However in transgenic recipients BLG-reactive cells were "silenced" presumably by microenvironmental expression of the BLG protein. These data are discussed in Chapter 8.

CHAPTER 7

ORAL TOLERANCE TO BOVINE BLG ADMINISTERED VIA VOLUNTARY INGESTION

Milk proteins are capable of inducing systemic tolerance when they are ingested as part of an animal's daily diet [330-334] or when they are present in drinking water or given by intragastric intubation [334]. Mice feeding on a solid diet in which either casein or whey proteins were the only protein source developed oral tolerance to these milk proteins. The onset of tolerance to dietary antigens is rapid since a 3 day exposure to casein and whey containing solid food diets resulted in 80-90% suppression of both antibody and T cell responses [331, 332]. T cell unresponsiveness to casein induced by oral tolerance was directed to the immunodominant and not cryptic components of this protein [332]. Oral tolerance was also observed to trypsin digested casein [330].
Despite the tolerability of milk proteins given orally, no tolerance to ovine BLG occurred in mice suckled on ovine BLG-containing milk at either the antibody (Chapter 4) or T cell level (Chapter 5). This suggested that a continuous oral challenge with ovine BLG postnatally was not sufficient to induce oral tolerance.
It was not possible to test the ability of BLG to induce oral tolerance in adult CBA/Ca mice due to a limited supply of purified ovine BLG. Instead CBA/Ca mice were given commercially prepared bovine BLG dissolved in their drinking water for both short and long periods of time to test the tolerogenic nature of this protein when administered in this fashion. Tolerance was investigated at both the antibody level, by ELISA, and the T cell level, by DTH assays.

7.1) Antibody Studies.

Groups of mice were given either 25mgs or 50mgs/day of bovine BLG dissolved in sterile water. Mice were either

TABLE 7.1. Bovine BLG feeding regimes

AMOUNT OF BOVINE BLG(mgs) *.	LENGTH OF EXPOSURE + SOLID FOOD	FEMALE ++ EXPTS	CONT	MALE ++ EXPTS	CONT
25	24 (W+)	2	2	2	2
50	24 (W+)	2	2	2	2
25	21 (W+)	2	2	2	2
50	21 (W+)	3	3	2	2
25	24 (W-)	2	2	2	2
50	24 (W-)	2	2	2	2
25	21 (W-)	1	1	1	1
50	21 (W-)	2	2	1	1

* Mice were provided with BLG in their drinking water at a concentration of 5mgs/ml and 10mgs/ml. The intake is calculated in the assumption that mice will drink approximately 5mls of water per day.
++= number of experiments, and the number of mice per group was usually about 5.

TABLE 7.2. % survival of CBA/Ca control mice after a second immunisation

MALE/FEMALE	W+/W-	% SURVIVAL
M	W+	64.3
F	W+	55
M	W-	30
F	W-	47.8

exposed for 24 hours or 21 days to this antigen. One half of the groups tested were on a diet known to contain bovine BLG (whey protein positive, W+) whilst the other groups were on a diet lacking this protein (whey protein negative, W-). The experimental regimens are summarised in Table 7.1.

Each group of mice were immunised 3 weeks following the end of exposure to bovine BLG with 50μgs of BLG plus FCA and bled 2 weeks later. Mice were immunised at this time with 2% heat aggregated BLG and bled a second time 7 days later. The antibody titres were measured by ELISA as described before and the titres for the primary and secondary immune responses of each mouse were plotted (see Figs 7.1, 7.2, 7.3 and 7.4).

Control CBA/Ca mice, used in the bovine BLG feeding experiments, were given sterile water to drink and were either on a (W+) or (W-) solid food diet. Regardless of food, more control CBA/Ca mice suffered anaphylaxis and died following a second immunisation with bovine BLG (2% Hag bovine BLG) than those mice given BLG in their drinking water. The number of control mice that died differed from experiment to experiment but was of the order of 50%. The percentage survival data are shown in Table 7.2. This made statistical comparisons between control groups and experimental BLG-fed groups in individual experiments sometimes difficult. To overcome this problem the combined anti-BLG antibody response of each bovine fed group were compared with the pooled anti-BLG titres for all control CBA/Ca mice tested, using a Mann Whitney test.

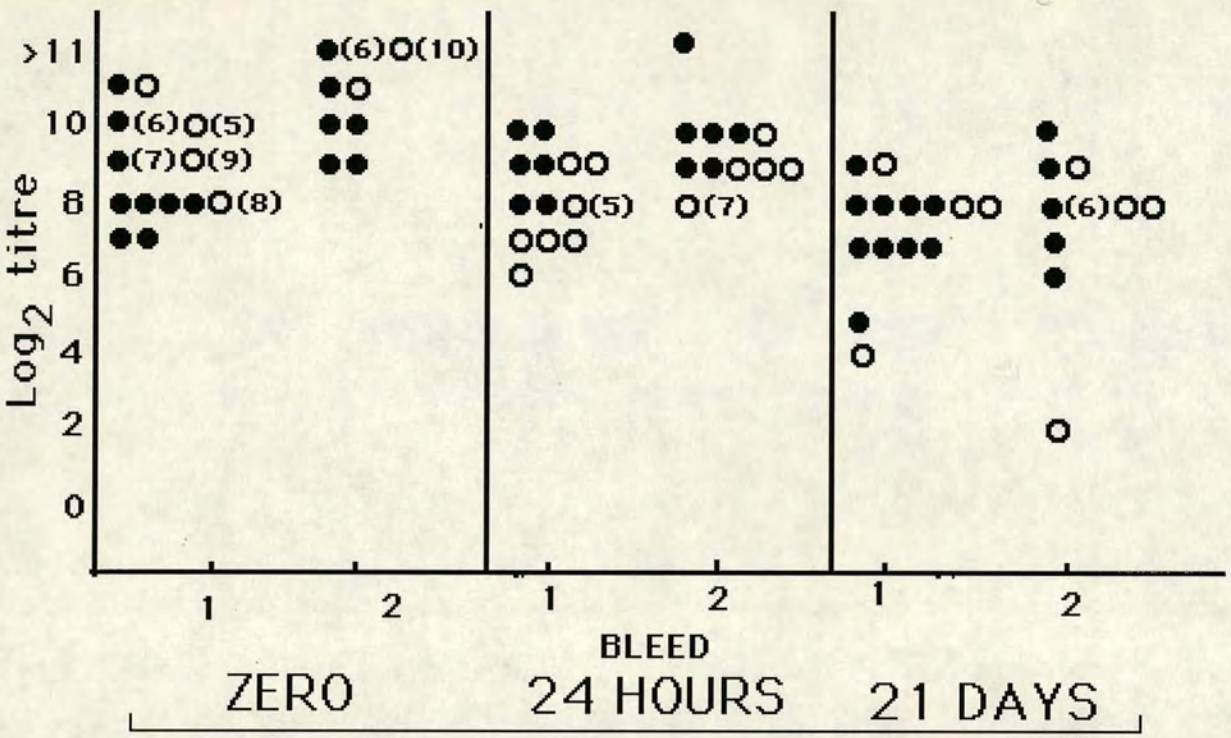
<u>i) Female mice fed Bovine BLG.</u>

Experiments were set up in which female CBA/Ca mice (on either the W+ or W- diet) were fed either 25 or 50mgs of bovine BLG for 24 hours or 21 days. The antibody responses of these mice following each immunisation with bovine BLG are shown in Figs 7.1 and 7.2, respectively.

The anti-BLG responses of BLG fed mice were compared with the anti-BLG responses of control mice to analyses the effect of feeding bovine BLG. The anti-BLG responses of mice fed for

Fig. 7.1.

The antibody responses of CBA/Ca females to bovine BLG. Each point is the data for serum from a single mouse.



Exposure time to bovine BLG in drinking water

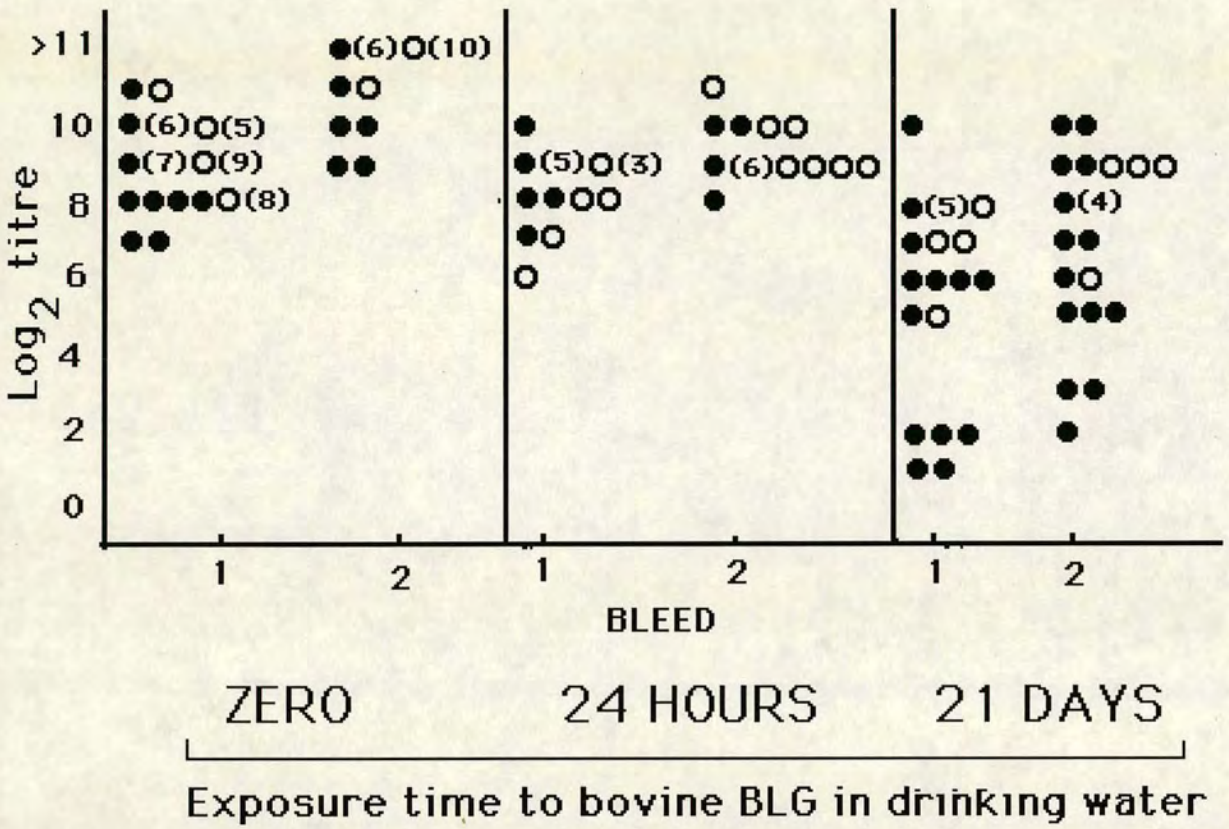
CBA/Ca female mice were fed either 25mgs of bovine BLG for 24 hours or 21 days or PBS. Three weeks after the end of the feeding regimen mice were immunised with 50µgs of bovine BLG+FCA. 2 weeks later mice were bled (1) and challenged with 2% Hag bovine BLG and 7 days post challenge they were bledagain (2)

● Mice fed on a diet containing whey protein (W+)
○ Mice fed on a diet lacking whey protein (W-)

(nos)= number of mice

Fig. 7.2.

The antibody responses of CBA/Ca female mice to bovine BLG. Each point is the data for serum from a single mouse.



CBA/Ca female mice were fed either 50mgs of bovine BLG for 24 hours or 21 days or PBS. Three weeks after the end of the feeding regimen mice were immunised with 50µgs of bovine BLG+FCA. 2 weeks later mice were bled (1) and challenged with 2% Hag bovine BLG and 7 days post challenge they were bled again (2)

● Mice fed on a diet containing whey protein (W+)
O Mice fed on a diet lacking whey protein (W-)
(nos)= number of mice

24 hours or 21 days were also compared to analyse whether feeding this protein for a long period of time induced a greater degree of hyporesponsiveness.

a) Comparison of the antibody responses in mice fed 25mgs of bovine BLG for 24 hours (25/24) or 21 days (25/21) (Fig. 7.1).

1) W+ mice

Both the primary and secondary anti-BLG responses of the 25/24 group were equivalent to saline fed control mice. In contrast the anti-BLG responses of the 25/21 group were significantly lower than control mice ($p<0.001$). Comparing the responses of 25/24 and 25/21 groups indicated that a longer exposure time to bovine BLG resulted in a greater degree of hyporesponsiveness following a primary ($p<0.05$) and secondary ($p<0.01$) immunisation.

2) W- mice

Comparing the primary anti-BLG responses of BLG fed mice and control mice indicated that the 25/24 group made a significantly lower response to this protein. This was not seen for the 25/21 group ($p<0.01$).

Regardless of the exposure period to BLG, the combined secondary anti-BLG IgG response of W- mice were lower than the equivalent responses of control mice ($p<0.001$).

Unlike the W+ mice a longer exposure time to bovine BLG did not result in a greater degree of hyporesponsiveness.

b) Comparison of the antibody responses of mice fed 50mgs of bovine BLG for 24 hours (50/24) or 21 days (50/21) (Fig. 7.2).

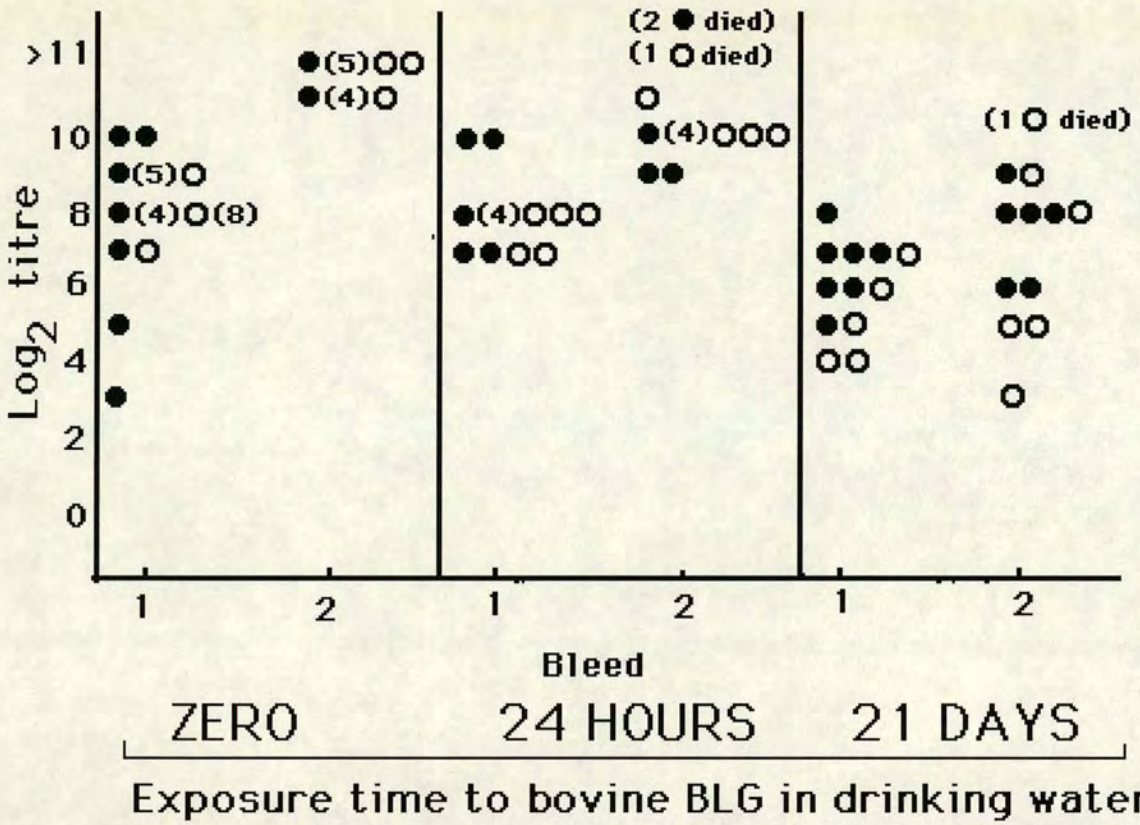
1) W+

Following a primary immunisation with bovine BLG, saline fed control and 50/24 mice made equivalent anti-BLG IgG responses. In contrast the primary responses of the 50/21 mice were significantly lower than control mice ($p<0.001$). Following a second immunisation both these BLG fed groups made significantly lower anti-BLG responses (50/24 $p<0.01$ and 50/21 $p<0.001$) than control mice. No difference was found between mice fed for 24 hours or 21 days.

1) W-

Fig. 7.3.

The antibody responses of CBA/Ca males to bovine BLG. Each point is the data for serum from a single mouse.



CBA/Ca male mice were fed either 25mgs of bovine BLG for 24 hours or 21 days or PBS. Three weeks after the end of the feeding regimen mice were immunised with 50ugs of bovine BLG+FCA. 2 weeks later mice were bled (1) and challenged with 2% Hag bovine BLG and 7 days post challenge they were bled again (2)

● Mice fed on a diet containing whey protein (W+)
O Mice fed on a diet lacking whey protein (W-)
(nos)= number of mice

As above, the 50/24 group made equivalent anti-BLG IgG following a primary immunisation with bovine BLG to control mice. In contrast the primary responses of the 50/21 mice were significantly lower than control mice ($p<0.01$). Following a second immunisation both these BLG fed groups made significantly lower anti-BLG responses (50/24 $p<0.001$ and 50/21 $p<0.001$) compared to controls.
Mice fed 50mgs of bovine BLG for 21 days made significantly lower primary and secondary anti-BLG titres than those mice fed for 24 hours ($p<0.01$ and $p<0.01$, respectively).

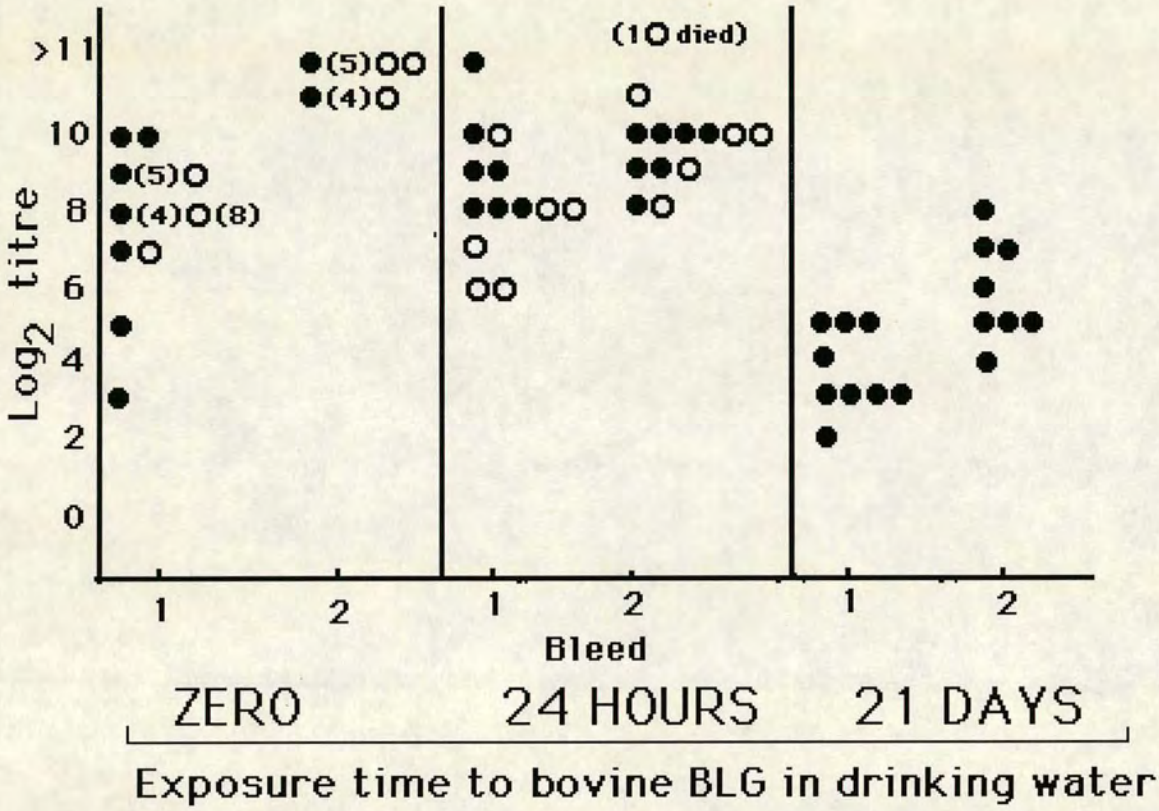
ii) Male mice fed bovine BLG

Male CBA/Ca mice were also tested and data analysed as described above.
a) Comparison of the antibody responses of mice 25mgs of bovine BLG for 24 hours (25/24) and 21 days (25/21) (Fig. 7.3).
1) W+
Following a primary immunisation only the 25/21 fed mice made significantly lower anti-BLG responses as compared to control mice ($p<0.05$). However following a second challenge both groups had reduced secondary anti-BLG responses as compared to control mice (25/24 $p<0.01$ and 25/21 $p<0.01$).
As with the female antibody data, giving bovine BLG for 21 days significantly reduced the primary ($p<0.05$) and secondary ($p<0.01$) anti-bovine BLG response as compared to feeding BLG for 24 hours.
2)W-
Following a primary immunisation only the 25/21 fed mice made significantly lower anti-BLG responses as compared to control mice ($p<0.01$) however following a second immunisation BLG fed and control mice made equivalent secondary anti-BLG responses.

ii) Comparison of the antibody responses of mice fed 50mgs of bovine BLG for 24 hours (50/24) and 21 days (50/21) (Fig. 7.4).
1)W+

Fig. 7.4.

The antibody responses of CBA/Ca males to bovine BLG. Each point is the data for serum from a single mouse.



CBA/Ca male mice were fed either 50mgs of bovine BLG for 24 hours or 21 days or PBS. Three weeks after the end of the feeding regimen mice were immunised with 50µgs of bovine BLG+FCA. 2 weeks later mice were bled (1) and challenged with 2% Hag bovine BLG and 7 days post challenge they were bled again (2)

● Mice fed on a diet containing whey protein (W+)
O Mice fed on a diet lacking whey protein (W-)
(nos)= number of mice

Giving mice 50mgs of bovine BLG for 21 days significantly reduced the primary anti-BLG responses in these mice ($p<0.001$). This did not occur in the 50/24 fed mice. Following a secondary immunisation with bovine BLG both the 50/24 and 50/21 fed mice made significantly lower anti-BLG responses than control mice ($p<0.001$ in both cases). The 50/21 fed mice made significantly lower responses than the 24 hour fed mice ($p<0.001$ and $p<0.01$ for primary and secondary antibody responses, respectively).

2)W-

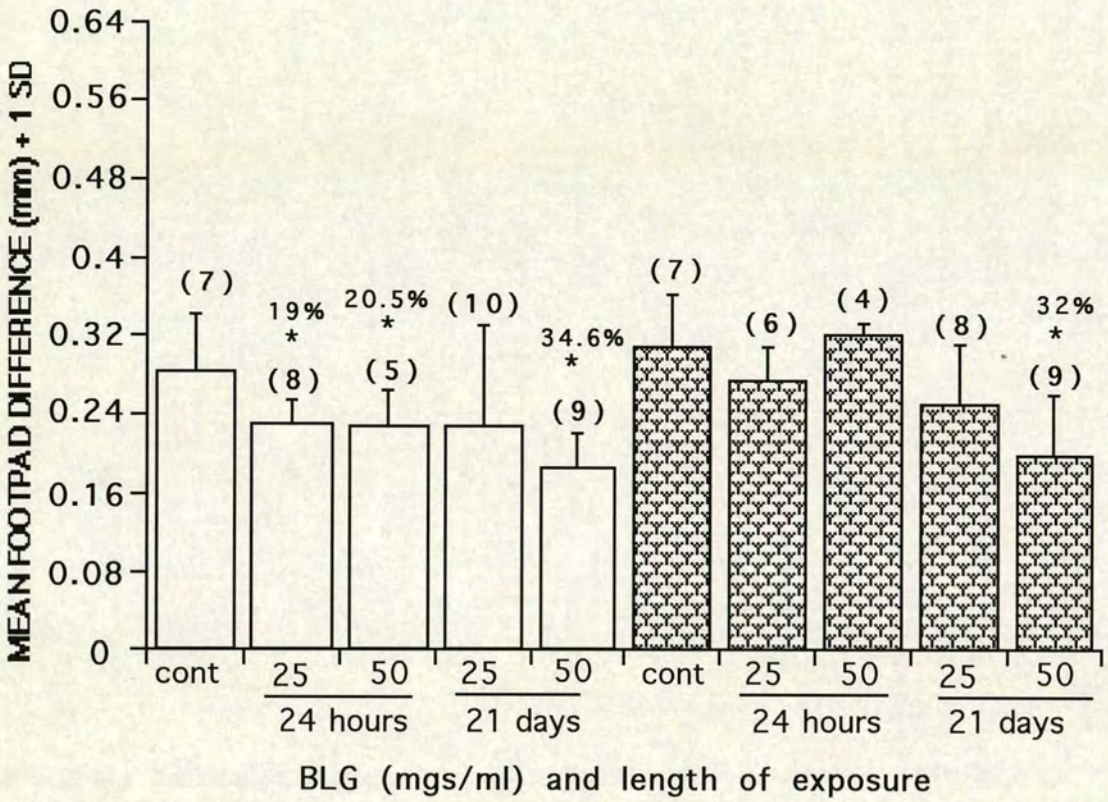
Male mice were fed bovine BLG for 24 hours. After a second immunisation the anti-BLG responses of these mice were significantly lower than control mice ($p<0.05$).

7.2) Summary of antibody data

Control mice that were not exposed to bovine BLG in their drinking water, respond to a second immunisation with this protein by producing antibody titres greater than the primary response ($p<0.001$). Although in some of the groups of mice given BLG orally (50/24 W- female, 25/24 and 50/21 W+ males) a significant ($p<0.05$, $p<0,05$ and $p<0.01$, respectively) increase in antibody titre followed a second immunisation, the titres of antibody never reached that of control mice. Thus introducing bovine BLG into the drinking water of CBA/Ca mice induced hyporesponsiveness to this protein, with the exception of the 25/24 W+ fed female mice. It was also noted that although a 24 hours exposure to BLG did induce hyporesponsiveness, a better suppression of the antibody response was seen after 21 days of oral administration. Both doses induced a similar level of suppression.

Although hyporesponsiveness at the antibody level to BLG could be detected after oral administration the degree of antibody reduction was small compared to that induced by the presence of the BLG transgene (see Chapter 4). In the light of these observations it is perhaps not surprising that differences between suckling or not suckling of BLG-containing milk were difficult to detect.

Fig. 7.5 T cell responses to bovine BLG of female CBA/Ca mice following voluntary ingestion of either 25 or 50mgs of bovine BLG.



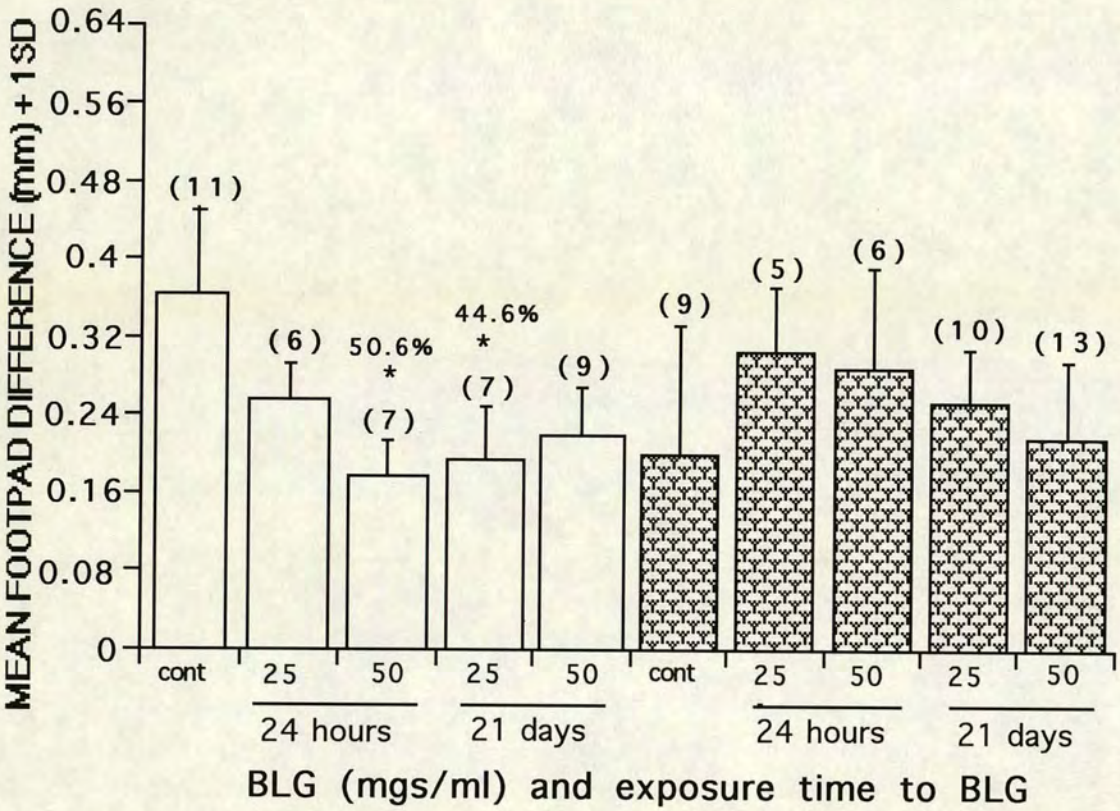
CBA/Ca mice maintained on a diet either containing (□) or lacking whey protein (▨) were fed 25 or 50mgs of bovine BLG for 24 hours or 21 days. Each group of mice were immunised 3 weeks following the end of exposure to BLG with 50μgs of bovine BLG+FCA. 2 weeks later mice were challenged with 2% Hag BLG.

(cont) = control mice received sterile water only
(nos) number of mice tested

* = significant suppression (% suppression)

$$\left(\%\ \text{Suppression} = \frac{\text{mean control} - \text{mean expt}}{\text{mean control}}\right)$$

Fig. 7.6 T cell responses to bovine BLG of male CBA/Ca mice following voluntary ingestion of either 25 or 50mgs of bovine BLG.



CBA/Ca mice maintained on a diet either containing (☐) or lacking whey protein (▨) were fed 25 or 50mgs of bovine BLG for 24 hours or 21 days. Each group of mice were immunised 3 weeks following the end of exposure to BLG with 50μgs of bovine BLG+FCA. 2 weeks later mice were challenged with 2% Hag BLG.

(cont) = control mice received sterile water only
(nos)= number of mice tested

* = significant suppression (%suppression)

7.3) T Cell Studies

The presence or absence of T cell hyporesponsiveness following administration of bovine BLG to drinking water was tested using the footpad thickening assay, as previously described (see Chapter 2). Individual T cell responses are shown in Appendix 16.

i) Comparison of the responses of female mice fed bovine BLG (Fig.7.5)

Although all W+ females fed bovine BLG mice had reduced T cell response to BLG only the 25/24, 50/24 and 50/21 groups were significantly hyporesponsive ($p<0.05$, $p<0.05$ and $p<0.01$, respectively) compared to control mice. The differences observed between these groups were however small and variable.

In W- females mice differences between control and fed mice T cell responses were similarly small and variable. Feeding 25mgs of BLG for 24 hours or 21 days resulted in a slight decrease in response but this was only significant following a 21 day exposure ($p<0.05$). Significantly reduced T cell responses were also seen after feeding 50mgs for 21 days group ($p<0.01$).

ii) Comparison of the responses of male mice fed bovine BLG (Fig. 7.6)

Although all W+ males fed bovine BLG had reduced T cell responses to BLG compared to controls a significant reduction in responsiveness was only observed following a 25mg/day bovine BLG fed over 21 days ($p<0.05$) and following a 50mg feed for 24 hours ($p<0.05$).

Like the female data the differences between experimental animals and controls were small and variable.

Unlike W+ fed mice, W- male mice exposed to BLG in drinking water made equivalent T cell responses to controls mice, the only exception being the 50/21 fed group.

7.4) Summary of T cell data

Although following exposure to bovine BLG in drinking water induced reductions in T cell responses to bovine BLG, as compared to controls, the differences were very small. This indicates that BLG is probably not an ideal antigen for investigating T cell tolerance.

CHAPTER 8

DISCUSSION

8.1) Immune responses to ovine BLG

Antigens can be described as either immunogenic or antigenic, the latter describing the property of an antigen to combine with surface or secreted antibodies but not to elicit an immune response. BLG is in fact antigenic since in both ELISA and Western blotting experiments, antibodies to BLG recognise this protein following purification. To be immunogenic an antigen must elicit either an antibody and/or a T cell response [335]. In this study purified ovine BLG induced both antibody and T cell responses (Chapter 3) after priming with an adjuvant, confirming its immunogenicity. BLG did not induce an immune response without an adjuvant (data not shown).

i) Antibody responses to BLG

Antibody responses to bovine BLG have previously been documented [336]. In these studies four antigenic regions on the molecule were described. 3 out of 4 of these regions were contained in the structure of a turn or random coil between β-sheets and all 4 epitopes were present on the outside of the three dimensional structure. Unfolding the BLG molecule did not reveal other B cell epitopes. The B cell epitopes described were ^{21}Ser-^{40}Arg, ^{149}Leu-^{162}Ile, ^{102}Tyr-^{124}Arg and ^{41}Val-^{60}Lys [336].

In this study the antibody responses to ovine and bovine BLG were measured using an ELISA technique. CBA/Ca, BALB/c, CBA/Ca x C57BJ/6 F1 and C57BJ/6 mice were shown to develop secondary antibody responses to ovine BLG. The level of secondary antibody responses to ovine BLG was similar to the antibody titres following an OVA challenge.

The immune response to bovine BLG was studied in CBA/Ca mice only. Good anti-BLG IgG antibody titres were observed

following secondary challenge with bovine BLG, confirming its immunogenicity (see above).
In conclusion, the protein BLG derived from either sheep or cows' milk can induce antibody responses comparable to those seen for OVA following a secondary challenge.

ii) T cell responses to BLG

T cell responses to BLG were analysed using a footpad thickening, DTH, assay. The response was quantitated by subtracting the footpad thickness (of BLG primed mice) before secondary challenge with the footpad thickness 24 hours following BLG challenge. Control mice (also primed with BLG) were challenged with PBS.
Development of a DTH response requires an initial sensitisation period of between 7 and 14 days. In this study the sensitisation period was either 7 days (Chapter 3 and 5) or 14 days (Chapter 7). Increasing the sensitisation period in Chapter 7 did not result in greater T cell responses.
Compared to control mice, good DTH responses were found to both ovine and bovine BLG in CBA/Ca mice and to ovine BLG in BALB/c, C57BJ/6 and F1 mice. It was also noted that the T cell response to ovine BLG was significantly lower than the response to bovine BLG. Why should there be such a large difference when both ovine and bovine BLG are cross-reactive proteins as shown at the antibody level (see Chapter 4). Takahashi et al (1988) [337] have investigated the proliferative responses of BLG primed lymph node cells from BALB/c mice to tryptic and chymotryptic fragments of bovine BLG. Bovine BLG T cell epitopes were located on the surface of the 3D molecule mainly in the β turn. These were ^{21}Ser-^{40}Arg, ^{41}Val-^{60}Lys and ^{102}Tyr-^{124}Arg from tryptic digests whilst ^{21}Ser-^{31}Leu, ^{59}Gln-^{82}Phe and ^{123}Val-^{140}Leu for chymotryptic fragments. T cell epitopes were similar to B cell epitopes [337].
4 bovine BLG specific T cell clones, with a CD4+ve phenotype, were isolated from BALB/c mice immunised with this protein. Two of these clones recognised a peptide containing residues

42-56 of bovine BLG. These residues along with residues 62-76 and 139-153 are the major T cell determinants of bovine BLG in BALB/c mice [338].

The nucleotide sequence of bovine BLG gene has recently been described [339]. The complete gene is 4724 base pair long and is 89% homologous to the ovine BLG gene sequence with complete conservation of gene organisation and splice sites [339]. In an earlier study Alexander et al (1991) [340] found that there was a 91% similarity between the cDNA sequences of ovine and bovine BLG [340]. In contrast it has been described that the primary structure of BLG from other species shows large diversity with an overall homology of only 13% of amino acids [341]. If these differences in ovine BLG lie within any of the above bovine BLG T cell epitopes this could explain the difference in responsiveness between bovine and ovine BLG.

Titus and Chiller (1981) [329] described footpad DTH responses to OVA, BSA and HGG. In these experiments BDF1 mice were primed with 50μgs of antigen subcutaneously at the base of the tail and challenged, 7 days later, with PBS in one foot and 2% heat aggregated antigen in the other footpad. The DTH response was measured by subtracting the footpad thickness following a PBS challenge from that following a heat aggregated challenge. This resulted in a 1.42, 2.15 and 1.82 millimetre increase following OVA, BSA or HGG challenges respectively. As discussed above increased footpad thickening occurred following an ovine or bovine challenge however responses were not as impressive as the T cell responses to the forementioned proteins. Why should this be? Perhaps priming T cells with BLG protein is not as efficient as priming with OVA, BSA or HGG proteins. Also in the aforementioned experiments the level of response was a measure of the difference between the thickness of the PBS injected versus the protein injected feet of the same mice. In the present study the response was a measure of the increase in the protein injected foot.

In conclusion, mice primed with either bovine or ovine BLG produce a secondary T cell response, as shown by the

measurement of a DTH response, compared to controls. Bovine BLG, however, appeared to induce a better T cell response than ovine BLG.

iii) MHC haplotype influence on the immune response to BLG

It has been shown that genes within the MHC can affect the response to a particular protein, see Chapter 3. Mice with different MHC backgrounds, H-2^k, H-2^b, H-2^d and H-2^{kxb}, were tested for both antibody and T cell responses to ovine BLG. All groups of mice, regardless of MHC background, made similar immune responses to ovine BLG (at the antibody and T cell level) and bovine BLG (at the T cell level).

These data suggest that segregation of MHC genes during backcrossing of transgenic animals was unlikely to have had an effect on the development of immunity to BLG.

8.2) The immune responses to BLG in BLG-transgenic mice.

i) Antibody responses

Both ovine and bovine BLG induce antibody responses in "normal" mice. This was not the case for mice carrying the BLG transgene. At the antibody level BLG-transgenic mice were hyporesponsive to both these proteins as compared to "normal" CBA/Ca mice and non-transgenic littermates. This immune hyporesponsiveness was not global since the responses of BLG-transgenic mice to a third party antigen, OVA, was similar to "normal" mice. Hyporesponsiveness could not be attributed to BLG gene expression during lactation since virgin male and female BLG-transgenic mice were tolerant to both bovine and ovine BLG. Subsequent pregnancy and lactation did not affect this tolerance since virgin and exbreeder BLG-transgenic female mice made comparably low antibody responses to ovine BLG.

Suckling on BLG-containing milk was also not the mechanism for this tolerance since BLG-transgenic male and female offspring suckled on "normal" mouse milk were as

hyporesponsive as those offspring suckled on BLG-containing milk.
It was concluded that tolerance was due to the possession and/or expression of the BLG transgene. This was confirmed by bone marrow chimaera experiments. Transferring bone marrow from CBA/Ca or non-transgenic mice into lethal irradiated transgenic mice did not abrogate antibody tolerance in the recipient mice. In contrast, transfering bone marrow from BLG-transgenic mice into lethally irradiated CBA/Ca or non-transgenic mice did not abrogate the responsive status of the recipient mice.
Although antibody hyporesponsiveness was observed in BLG-transgenic mice a 100% antibody suppression level was never achieved. Recent findings indicate that tolerance to immunodominant epitopes of HEL but not to the subdominant epitopes of this molecule can occur [342]. Low levels of antibody in the BLG-transgenic mice might be to the subdominant epitopes. It is also possible that the antibody responses were to other milk proteins contaminating purified BLG samples (see Chapter 2).

ii) T cell responses

Unlike the antibody data, T cell hyporesponsiveness to either bovine or ovine BLG was not evident. BLG-transgenic mice made comparable T cell responses in vivo and in vitro to both these proteins as compared to CBA/Ca mice and non-transgenic littermates, although the attempts to detect T cell tolerance to this protein were against a background of intrinsically low normal responses.
The role of T cells in the hyporesponsiveness at the antibody level will be discussed below.

8.3) Peripheral expression of milk proteins.

If T cell tolerance is responsible for the observed antibody hyporesponsiveness in the BLG-transgenic mice then the question arises as to where the BLG-transgene was expressed. Transgene expression in either the thymus or in peripheral

tissues, such as the liver or kidneys, has been shown to induce tolerance in transgene specific T cells (see Chapter 1).
Various groups have created transgenic mice expressing milk proteins (rat and goat β casein, cow and guinea pig α lactalbumin, and mouse and rat WAP) under the control of their own 5' promoter and 3' flanking regions, or non-milk proteins; α1 antitrypsin, human a) tissue plasminogen activator, b) HG, c) oncogene products, d) serum albumin, e) IFNγ and chloroamphenicol acetyl transferase (CAT)) under the control of 5' promoter regions of various milk protein genes. Although expression of these proteins should have been tissue specific, i.e. restricted to mammary gland tissue expression in other tissues was observed (see Table 8.1).
Analysis of the peripheral expression of BLG in various BLG-transgenic lines [293], carrying the native BLG gene and 4.3kb 5' promoter and 7.3kb 3' flanking regions, has shown that BLG mRNA was present only in the mammary glands of BLG-transgenic mice, at low levels in virgin mice [310] and not in the kidneys, spleen, liver and lachrymal glands of lactating mice. In my study no BLG mRNA expression was evident in the lymph nodes of virgin BLG-transgenic mice. Barashi et al (1994) [343] also created ovine BLG transgenic mice. These mice expressed the native ovine BLG gene under the control of either a 3 or 5.5kb BLG promoter along with 3' flanking regions. They also found that BLG mRNA expression was limited to the mammary gland tissue and that brain, kidney, skeletal muscle, skin and salivary gland tissue of virgin and lactating transgenic female mice were negative for the transgene expression.
Therefore in contrast to transgenic mice expressing 'foreign' WAP, casein and α lactalbumin, the BLG transgene, under the control of its own 5' promoter and 3' flanking regions, is tissue specific, i.e. restricted to the mammary secretory epithelial cells [310]. These data suggest that the BLG gene construct used to generate the mice in my study appears to harbour a unique set of regulatory elements that results in mammary-specific expression.

TABLE 8.1

MILK TRANSGENE PROMOTER	THYMUS EXPRESSION TESTED	NON-MAMMARY TRANSGENE EXPRESSION	METHOD OF TRANSGENE DETECTION	REF
Rat WAP	+	Kidney	RNAase protection	Bayna and Rosen (1990)[368]
Bovine α lactalbumin	-	Testis (male) liver, kidney, salivary gland, spleen and lachrymal gland (female)	Northern Blotting	Vilotte (1989)[369]
Rat βcasein	+	Brain	RNAase protection	Lee (1988)[370]
Guinea pig α lactalbumin	+	Skin , salivary gland, heart, lung(lactating female)	Northern Blotting	Maschio (1991)[371]
Ovine BLG	-	NONE	Northern Blotting	Barashi (1994)[343]
Ovine BLG	-	NONE	Northern Blotting	Simons (1987)[293]
Ovine BLG	-	NONE	Northern Blotting	Harris (1990)[311]
Goatβcasein	+	Skeletal muscle and Brain of lactating females	Northern Blotting	Roberts (1992)[372]
Goatβcasein	+	skin (lactating female)	Northern Blotting	Persy (1992)[373]
Murine WAP	+	Salivary gland	Northern Blotting	Wall (1991)[374]

However in contrast to the "native" BLG-transgenic mice, ectopic expression of the human serum albumin (HSA) gene under the control of either 3, 5.5, 10.8kb of 5' BLG promoter regions has been shown [343]. Lactating BLG/HSA transgenic females express HSA in their milk. In these mice the BLG/HSA construct was not exclusively expressed in the mammary gland secreting epithelial cells but was also present in skeletal muscle, kidney, brain, spleen, salivary gland and skin of lactating transgenic female mice. Mice that did not produce HSA in their milk did not have ectopic expression. Unlike female mice, HSA transcripts were found in the kidney or the salivary gland of 2 lines of transgenic male mice suggesting that this ectopic expression was hormonally regulated.
Ectopic expression of proteins under the control of milk protein 5' promoter regions is not unique to the BLG/HSA transgenic mice, see Table 8.1. Ectopic expression of supposed tissue specific proteins has been explained as being due to the interaction of the elements of the BLG (or other milk protein) 5' flanking promoter region and the HSA (or other proteins) sequences, resulting in a unique pattern of expression that differed from the original genes. It also suggests that the 3' flanking region and the BLG gene itself were important for mammary specific expression.

8.4) Thymic expression of milk proteins

Some authors (Table 8.1) analysed transgene expression in the thymus of their transgenic animals via Northern blotting and RNAase protection assays. Thymic expression was only found in two cases. Lee et al (1989) [354] found thymic expression of CAT in the thymus of mice expressing CAT under the control of the rat β casein regulatory elements, whilst Husbands et al (1992) [196] and Sponaas et al (1994) [176] found thymic expression of H-2K^{b} under the control of the 5' flanking regions of guinea pig α lactalbumin. Thymic expression of a transgene under the control of non-milk promoters, for example, liver [358] or pancreas [359] specific promoters, has also been shown via Northern blotting. These models suggest

TABLE 8.1 CONT

MILK TRANSGENE PROMOTER	THYMUS EXPRESSION TESTED	NON-MAMMARY TRANSGENE EXPRESSION	METHOD OF TRANSGENE DETECTION	REF
BLG/α1 antitrypsin	-	Salivary gland	Northern Blotting	Archibald (1990)[375]
BLG/HIFNγ	+	NONE	Northern Blotting	Dobrovolsky (1993)[347]
BLG/HSA	-	Skeletal muscle and kidney	Northern Blotting	Shani (1992)[376]
BLG/HSA	-	Brain, spleen, salivary gland, skeletal muscle, kidney, skin of lactating females	Northern Blotting	Barashi (1994)[343]
Guinea pig α lactalbumin /H-2K^b	+	Skin and thymus	RNAase protection	Husband (1992)[196]
Rat β casein/CAT	+	Thymus of virgin and lactating females	CAT assays	Lee (1989)[344]
Murine WAP/HaRas oncogene	-	Brain	RNAase protection	Andres (1987)[377]
Murine WAP/HHG	-	Bergman cells in the brains of lactating females	Northern Blotting	Grunzburg[378] (1991)
Murine WAP/Human tissue plasminogen activator	+	Tongue, kidney, subingual gland in lactating females and virgin male and females	RNAase protection	Pittius (1988)[379]

that the tissue-specificity of a transgene is sufficiently 'leaky' to allow thymic expression.

Other authors did not investigate whether transgene expression was evident in the thymus of their transgenic animals (see Table 8.1 cont). For example Barashi et al (1994) [343] did not investigate thymic expression of BLG, or BLG/HSA in "native" BLG and BLG/HSA transgenic mice and thymic expression was also not originally analysed in the ovine BLG-transgenic mice, carrying the native BLG gene (4.3kb 5' promoter and 7.3kb 3' flanking regions) used in my study [293]. Although no thymic expression of BLG/HIFNγ was found [352] it is possible that thymic expression of BLG might occur since mammary-specific transgene expression is leaky, as described above, and since thymic expression of the VSV glycoprotein (VSV-GP) was found in BLG/VZV-GP transgenic mice (Steinhoff et al, unpublished data)

In the present study, thymus tissue from virgin male and virgin and lactating female BLG-transgenic mice was analysed for BLG mRNA expression. Total RNA was extracted from these tissues and, following gel electrophoresis and Northern blotting, probed with ^{32}P labelled BLG cDNA [293]. No thymic expression of BLG mRNA was observed in any of these tissues, even after long autoradiograph exposure. Thymic expression of BLG protein was also not found (data not shown) and taken together these data indicate that BLG is not expressed intrathymically. It is however possible that these assays are not sensitive enough to detect very low levels of the BLG-transgene expression in the thymus. Low levels of the T antigen (Tag) mRNA, under the control of the rat insulin promoter, were found in the thymus of Tag transgenic mice even though expression was directed to the pancreas [360]. Low levels of the transgene expression, 3000-10,000 times lower than that of pancreatic B cells, were revealed using RT-PCR, a more sensitive technique than other RNA detection systems. Future experiments should include RT-PCR assays of thymic RNA isolated from the both BLG-transgenic and CBA/Ca control mice.

Husbands et al (1992) [196] were able to confirm thymic expression of H-2K^b in mice transgenic for this protein through the use of thymic chimaeras. Thymectomised H-2K^k mice were given T cell depleted H-2K^k bone marrow and either an H-2K^k or an H-2K^b, non-transgenic or transgenic adult thymus, respectively, under the kidney capsule. 90 days later mice were given a H-2K^b skin graft. Only those mice given the H-2K^b transgenic thymus accept the graft. A similar experiment could be set up using the backcrossed BLG-transgenic and non-transgenic mice except the test for tolerance would be the absence of BLG-specific antibody following immunisation. This experiment could be simplified by using CBA/Ca nude mice given 2' deoxyguanosine treated transgenic and non-transgenic thymi under the kidney capsule such that host derived T cells developed in the donor thymus. Again the test of tolerance would be the absence of BLG-specific antibody following immunisation.

Is tolerance to BLG due to peripheral expression of this transgene? Soluble peripheral antigens can reach the thymus [162, 342, 361] and either induce tolerance [162, 342] or have no effect [361]. Low doses of HEL (blood levels >10ng/ml) induced T cell tolerance to HEL and the immunodominant HEL peptide whilst 200ngs/ml of soluble H-2K^k did not. Tolerance therefore not only depends on the amount of protein in the serum but on the nature of the protein. The experiments in my study do not rule out the possibility that small amounts of soluble BLG reach the thymus where they may or may not have an effect. The RNA data however indicate that circulating protein concentrations would be extremely low.

8.5) T cell tolerance hypothesis

i) T_H1 vs T_H2

BLG was described earlier as a T dependent antigen, implying that B cells required T cell help before they could produce antibody to this protein. The lack of antibody but not T cell responses to both ovine and bovine BLG in the BLG-transgenic mice suggests that no T cell tolerance had occurred. However

it is possible that differences in tolerogenic susceptibility occurred at the level of CD4+ve T helper cell subsets in these mice. For example, T_H2 cells but not T_H1 cells may have been tolerised in these transgenic mice.

T_H1 cells have been described as the T helper cell subset responsible for in vivo DTH responses [362] and in vitro lymph node proliferation following a subcutaneous challenge with FCA plus antigen [363]. Since T cell responses in my study were measured using in vivo DTH responses and in vitro lymph node proliferation assays, following subcutaneous priming with BLG plus FCA, it is possible that the T cell responses noted in BLG-transgenic, CBA/Ca and non-transgenic mice were due to T_H1 priming. It is also possible that in the transgenic mice this helper T cell subset was refractory to tolerance induction.

Priming of T_H2 cells, on the other hand, occurs following immunisation with an antigen in Alum. For example, CBA/H mice given primary and secondary immunisations with 50μgs of BSA in Alum produced IgG1 antibodies [364] characteristic of a T_H2 response. Since immunisation with BLG plus Alum resulted in the absence of a T cell dependent antibody response in BLG-transgenic mice this T helper cell subset maybe susceptible to tolerance.

Differences in the susceptibility of CD4 subsets to tolerance induction have been published [365-367]. The experiments in these papers suggest that it is the T_H1 and not the T_H2 cells that are susceptible to tolerance: however this conclusion is derived from experiments in which tolerance was induced peripherally. In my experiments any possible T_H2 tolerance would have to be either due to thymic or unspecified expression of the transgene.

Immunisation (ip) or (iv) with either deaggregated soluble human gamma globulin, dHGG, [367] or UV-inactivated Theiler's murine encephalomyelitis virus [365, 366], respectively, resulted in antigen-specific unresponsiveness of the T_H1 cells as shown at the cytokine level. In contrast priming of T_H2 cells was observed [366]. Differences in tolerance

susceptibility at the T_H subset level were also described by Gilbert et al (1990) [368]. Following in vitro stimulation with fixed APCs, that had been preincubated with HGG (to allow processing and presentation of HGG peptides) T_H2 cells but not T_H1 cells made proliferative responses to HGG presented by freshly isolated APCs. Although the response of T_H2 cells was not affected, their ability to support B cell antibody production was tolerised. This may explain why T cell responses following an BLG/Alum subcutaneous injection were found despite the lack of antibody production. Although Gilbert et al (1990) [368] ruled out the possiblity that T_H1 and T_H2 cells recognised different HGG epitopes, since $V\beta$ expression on HGG specific T_H clones were markedly heterogeneous, the possibility that T_H1 and T_H2 cells recognise different epitopes of bovine and ovine BLG can not be ruled out.

Although T_H1 and T_H2 cells participate in antibody production, it is possible that the response to BLG is predominantly a T_H2 phenonomen. Cher and Mossman (1987) [362] have suggested that some antigens appear to preferentially induce one or other or both T_H1 and T_H2 cells. In the present study the antibody isotype profile was not analysed so it is not known which T cell subset(s) were induced following BLG immunisation. Future experiments investigating the isotype profile, particularly concentrating on the IgG1, T_H2 antibody responses verses IgG2a, T_H1 antibody responses, of the immune response to BLG in CBA/Ca, non-transgenic and BLG-transgenic mice would help to clarify this point. If the above hypothesis was correct the predominant class of antibody produced following a secondary challenge in CBA/Ca and non-transgenic mice would be IgG1, while the antibody profile for BLG-transgenic mice would lack this isotype, regardless of the adjuvant used during challenging.

Hyporesponsiveness at the antibody level was not 100% in the BLG-transgenic mice. It is possible that this is due to the priming of non-tolerant T_H1 cells following an Alum challenge. Bomford (1980) [364] found that the secondary response of

BSA plus Alum resulted in both IgG1 and IgG2a responses. Again, future experiments would investigate this point.
Although the above are attractive theories preliminary experiments have shown that BLG-transgenic mice immunised with ovine BLG plus FCA, intraperitoneally, remained hyporesponsive at the antibody level as compared to non-transgenic mice (data not shown). Although this supports the hypothesis that T cells are not rendered hyporesponsive in the BLG-transgenic mice it also supports the hypothesis that T cell tolerance in these mice may be at the T_H2 subset level since immunisation with FCA may not stimulate T_H2 cells involved in the response.

ii) Possible mechanisms of T cell tolerance.

Thymic involvement in the antibody hyporesponsiveness to BLG in BLG-transgenic mice cannot be ruled out. If tolerance was achieved at the level of the thymus what mechanism(s) would be involved? Tolerance in the thymus has been attributed to deletion of self-reactive cells through expression of a self antigen on thymic stromal cells. Anergy can also occur when antigen is encountered on these cells. Thymic epithelial and bone marrow-derived cells have also been shown to induce tolerance via deletion [191-193], anergy [177, 189] and down regulation of TCR and CD8 molecules [196, 197]. Recent work suggests that thymic epithelial cells can also induce tolerance to Class 2 presented antigens.
Husbands et al (1992) [196] created transgenic mice expressing H-2K^b under the control of the guinea pig α lactalbumin promoter elements. These mice were tolerant to this alloantigen, tolerance being attributed to thymic expression of the H-2K^b transgene. Sponaas et al (1994) [176], through the use of bone marrow chimaeras, indicated that unlike bone marrow-derived cells, radioresistant cells (presumably epithelial cells) in the thymus of these mice were responsible for this tolerance. Crossing these mice with H-2K^b specific TCR transgenic mice indicated that tolerance occurred late in development through deletion of self-reactive

cells since large numbers of DP cells expressing the anti-H-2K^b transgenic receptors were present whilst CD8+4- TCR transgenic cells were absent. Deletion of these cells was attributed to transgene expression in the medullary epithelial cells [176].

Bone marrow chimaera experiments in this thesis also agree with the above bone marrow chimaera findings; that transgenic radioresistant cells rather than bone marrow-derived cells are responsible for tolerance since transfer of BLG-transgenic bone marrow into either lethally irradiated non-transgenic littermates or CBA/Ca mice resulted in no antibody tolerance. Transferring non-transgenic or CBA/Ca bone marrow into lethally irradiated BLG-transgenic mice resulted in tolerance. It is possible that developing non-transgenic T cells are rendered tolerant to BLG expressed on thymic epithelial cells. Deletion of BLG-reactive T cells could not be analysed in the present study. CD4+ve T cell clones that recognise bovine BLG have been isolated from immunised BALB/c mice [338]. Sequencing TCR genes of these clones may facilitate the production of anti-BLG TCR transgenic mice which would allow the derivation of double transgenic animals with which to analyse thymic tolerance to BLG.

The expression of a self protein on thymic epithelial cells has also been shown to induce a state of 'split-tolerance' [194, 195]. Split-tolerance results in unresponsiveness in vivo but not in vitro. If thymic epithelial cells are responsible for the BLG-transgenic T cell tolerance, 'split-tolerance' may also occur in these mice. Split-tolerance might result in the deletion of high affinity T_H2 cells, which might explain the reduced anti-BLG responses in these mice, whilst low affinity T_H1 cells escape tolerance, which might explain the T cell in vitro activation following BLG stimulation.

Tolerance to transgenes outwith the thymus has been documented and is known as peripheral tolerance. Can systemic tolerance occur as a result of specific expression of the BLG transgene in the mammary gland secretory cells? To date tolerance induced by exclusive transgene expression in

the mammary gland has not been documented. Peripheral tolerance, even after in vivo priming, to MHC class 1 and 2 as well as viral protein transgenes expressed in liver, pancreas, erthyroid, neuroectodermal or epithelial cells (see Chapter 1) however has been established. These data suggest that peripherally expressed transgenes were both accessible to the immune system and tolerogenic. Virgin and pregnant BLG-transgenic mice express BLG mRNA at either very low or high levels in the mammary gland respectively. Although sections of mammary tissue from both virgin and lactating BLG-transgenic mice were not tested for T cell infiltrates, the lack of tissue damage and the absence of mammary tumors suggests that at both low and high levels of expression, the BLG-transgene did not induce any autoimmune infiltration. The absence of an autoimmune response in the mammary tissue is not due to this organ being an immunologically privileged site since; 1) following infusion of sheep mammary glands with killed Staphylococcus aureus or OVA Class 2 +ve B cells, CD4 +ve and CD8+ve cells were found [369, 370] and 2) lymphocytes from the Peyers' patch have been shown to migrate to the mammary gland with an influx of IgA-producing cells during late pregnancy and early lactation [371].

If tolerance to BLG is a peripheral phenonomen the mechanism of tolerance is unknown. It is possible that BLG produced in the virgin mammary gland is taken up by non-professional antigen presenting cells (APCs) surrounding the mammary tissue which home to the local lymph node. Presentation of antigen by non-professional APCs may induce tolerance via anergy. The absence of any detectable protein and extremely low or absent levels of mRNA in virgin female and male transgenic animals make this explanation unlikely.

8.6) B cell tolerance hypothesis

An alternative explanation for the described antibody hyporesponsiveness is that tolerance is at the level of the B cell. B cell tolerance to proteins has been documented (see

Table 8.2 B cell tolerance

ANTIGEN	FORM OF ANTIGEN (promoter)	B CELLS PRESENT/ RESPONSIVE	T CELLS HELP
VSV-G	Membrane (SV40)	+/-	-
VSV-G	Membrane (H-2K^b)	+/-	-
H-2K^k	Membrane	-/-	-
HEL	Soluble	+/-	-
HEL	Membrane	-/-	-

Chapter 1) although this tolerance has been found to co-exist with T cell tolerance (see Table 8.2). One example of this is described in experiments with soluble HEL transgenic mice. Immunising these mice with HEL coupled to SRBC resulted in reduced (by 50 fold) anti-HEL responses whilst anti-SRBC responses were similar to that of controls. Since T cells in these mice were tolerant to HEL coupling HEL to SRBC provided alternative T cell help to HEL-specific B cells.

Recently B cell tolerance in the absence of T cell tolerance has been documented [372]. Ryelandt et al (1995) [372] noted that offspring suckled on dHGG-containing mother's milk did not produce anti-HGG-specific antibodies when challenged with the immunogenic forms of HGG plus FCA in adulthood. In contrast anti-HGG-specific T cells responses occurred following immunisation with either the immunogenic forms of HGG or with haptenated HGG. Non-tolerant HGG specific T cells produced a cytokine profile characteristic of both a T_H1 and T_H2 response. B cell tolerance in the absence of T cell tolerance was confirmed by experiments where T cells had been depleted via anti-CD3 treatment. B cell tolerance to dHGG occurred in the absence of T cells.

B cell tolerance occurs through either deletion or anergy, the general consensus being that clonal deletion occurs when B cells encounter membrane bound (multivalent) antigen whilst functional silencing or anergy, occurs when B cells encounter soluble (monovalent) antigen. If B cell tolerance does exists in BLG-transgenic mice which of these mechanisms results in tolerance? Anergy has been shown to occur when self-reactive B cells encounter large concentrations of soluble self antigen, for example HEL (see Chapter 1). No evidence for soluble BLG was found in these mice (data not shown) making the observed tolerance unlikely to be via anergy. However anergy can occur following interaction with membrane proteins which are at low density, for example VSV-GP (see Chapter 1). Mice transgenic for VSV-GP are tolerant at the antibody level perhaps due to transgene expresson in the kidney, heart, thymus, brain, liver and CNS. As mentioned earlier no

expression of BLG on various tissues was evident although it is possible that these tests were not sensitive enough to detect very small amounts of protein. It has also been demonstrated via irradiation chimaera studies that anergy is an intrinsic property of the tolerant B cells such that tolerance is transferable. In my study tolerance was not transferable, although bone marrow rather than spleen cells were being transferred.

B cell tolerance in the BLG-transgenic mice maybe attributed to deletion of BLG-specific B cells. Clonal deletion of B cells occurs when immature B cells encounter antigen in the bone marrow; for example, membrane bound H-2K^k and membrane HEL induce the deletion of anti-H-2K^k-specific B cells and anti-HEL -specific B cells respectively (see Chapter 1). If B cells are being deleted in BLG-transgenic mice it is possible that this is a result of BLG transgene protein expression within the marrow stroma. However no data on BLG mRNA expression in the bone marrow is available to date. Deletion has also been shown using bone marrow chimaera experiments. For example, transferring bone marrow from mice expressing transgenic anti-H-2K^k Ig into H-2K^k mice resulted in deletion of anti-H-2K^k specific B cells. Transferring bone marrow from anti-HEL mice into HEL expressing mice resulted similarly in deletion of HEL-specific B cells. In my bone marrow chimaeras B cell tolerance occurred if haematopoietic cells were placed in a BLG-transgenic host. This result is similar to those described above suggesting that the absence of a BLG-specific antibody response maybe due to deletion of B cells within the bone marrow.

Published data on B cell tolerance has relied on transgenic mice in which all B cells expressed a particular antigen specific idiotype (see Chapter 1). Unlike the B cells in these mice, B cells in the BLG-transgenic mice are not a monoclonal population making investigation of the tolerogenic mechanism difficult.

8.7) Is BLG an oral tolerogen?

Experiments in which newborn mice were exposed to antigens orally during the neonatal period have suggested that priming rather than tolerance occurs following exposure to an antigen in the first few days of life [262, 373-375]. Adult mice fed OVA (1mg/g of body weight) on the first day of life made enhanced antibody responses to OVA following parenteral immunisation compared to saline fed controls [262, 374]. Tolerance was established if mice were fed OVA from day 7 onwards [374] or after day 10 of life [262]. Continuous exposure however from days 1-4 or 1-14 [262] resulted in suppression of both antibody and T cell responses; continuous exposure affecting the T cell more than the antibody response. HGG has also been shown to induce effective oral tolerance [374, 376]. In contrast to OVA no priming occurred when similar doses of HGG (1mg/g of body weight) were fed to 1 day old mice: a reduction in the dose to 1μg/g was necessary to produce this effect [374].

In the above experiments antigen was administered through an intragastric tube. Oral tolerance in neonatal mice has also been shown through a more "natural" system' i.e. suckling antigen contained in their mother's milk [374, 376, 377]. Deaggregated HGG [374, 376] and deaggregated OVA [374] were injected into female mice 24 hours [374, 376] or 3 days postnatally [374]. Tolerance to both proteins, as seen by the lack of antibody responses in suckled mice following parenteral immunisation, was observed [374, 376]. Tolerance in these cases was due to the absorption of antigenically intact protein after suckling, since 0.3-0.6mgs/ml of HGG was found in neonatal circulation. Tolerance was also specific since HGG-suckled mice responded to a non-specific antigen (bromine treated RBC) to the same extent as non-suckled mice. Tolerance to sheep RBCs has also been shown in mice suckled on RBC containing mother's milk [374].

Taken together these data suggest that oral tolerance can occur in neonatal mice either fed antigen intragastically or through suckling.

Oral administration of protein to adult mice also results in systemic tolerance. For example, 25mgs of OVA dissolved in saline, administered intragastrically resulted in up to 93% IgG suppression [266, 288] and 97% T cell suppression [266, 288] The dose of antigen given has been shown to be important [290]: a 5-25mgs OVA feed induced both DTH and anti-OVA hyporesponsiveness whereas a 1-2mg OVA feed resulted in significant antibody but not DTH suppression in BALB/c mice. A low dose of OVA, 100 or 500μgs, suppressed neither the antibody nor the T cell responses, whilst 10μgs resulted in priming of the DTH, but not antibody responses.

OVA administered orally is taken up rapidly by the gut [261, 266, 287]. Sera taken 5 and 60 minutes from mice fed 25mgs of OVA, contained 44ngs/ml and 74ngs/ml of OVA respectively [287]. Injecting sera taken 5 minutes following an OVA feed into naive recipient mice did not affect the antibody or DTH responses to injected OVA in these mice. On the other hand sera taken after 60 minutes suppressed DTH responses to OVA [287]. These data suggested that processing of an antigen is important for tolerance induction.

Ovine BLG-transgenic mice producing a foreign protein in high concentrations in milk, allowed the effect of administering protein through a "natural" oral route to be analysed. Crosses of BLG-transgenic X CBA/Ca mice were set up such that both transgenic and non-transgenic offspring were suckled on either BLG-containing milk or "normal" mouse milk from lactating BLG-transgenic or CBA/Ca mothers respectively. Offspring were suckled on their mothers milk for 21 days before being weaned onto a diet containing or lacking bovine BLG. As mentioned above continuous feeding of OVA from day 1-14 of life and suckling on HGG containing milk resulted in antibody and T cell tolerance. It was thus hypothesised that continuous exposure to BLG, for 21 days, would have the same effect. Mice were challenged at 3 months of age with ovine BLG plus Alum intraperitoneally for antibody responses or with ovine BLG plus FCA subcutanously for T cell responses.

Although significant systemic hyporesponsiveness, at both the antibody and T cell level, occurred following oral administration of bovine BLG to adult CBA/Ca mice (see below), no evidence for oral tolerance to ovine BLG was observed in mice suckling on ovine BLG-containing milk. The antibody responses in both non-transgenic mice suckled on either BLG-containing or "normal" milk made equivalent responses with the exception of non-transgenic females in one experiment only (Chapters 4 and 5). Suckling BLG-containing milk did not affect the hyporesponsive seen for BLG-transgenic mice. At the T cell level, as measured by DTH responses, no tolerance was found; non-transgenic mice suckled on either BLG-containing or "normal" milk made equivalent DTH responses to BLG.

Although mice were exposed to ovine BLG within the first 24 hours of life no priming of either the antibody or T cell responses was evident. This was further shown via cross-fostering experiments. Neonatal mice were suckled on BLG-containing milk or "normal" mouse milk for the first 4-6 days of life before being cross-fostered on to lactating mother producing "normal" mouse milk or BLG-containing milk respectively. In this experiment (data not shown) exposure to BLG early in life did not result in either priming or tolerance.

Why did the exposure of neonatal mice to ovine BLG for the first 21 days of life not induce oral tolerance at both the antibody and T cell level? Oral tolerance in adult animals, to whey proteins, present as the major protein component of a solid food diet, has previously been reported [331] in BALB/c mice and this finding was confirmed for CBA/Ca mice in Chapter 7. Mice fed bovine BLG at concentrations in the range of 25-86mgs/ml (Chapter 7) or 290mg per day had a 5-7 fold reduction in anti-BLG IgG responses and a 35-45% reduction in anti-BLG T cell responses or a 98% suppression of both antibody and T cell responses respectively. Taken together these data suggest that BLG is tolerogenic. However its tolerogenic properties may only be revealed following feeding with a large quantity. Perhaps then the amount of BLG ingested

by neonatal animals was not sufficient to induce tolerance. Although the amount of ovine BLG ingested by neonatal mice was not calculated, BLG was shown to be present in the stomachs of pups suckling on BLG-containing milk. Electrophoresis of the gut contents of mice suckled on BLG-containing milk showed a strongly staining band after Coomassie blue staining and Western blotting of an intensity that suggested BLG was the major protein component in this milk. This finding is in agreement with Simons et al (1987) [293] who found that BLG was the major protein in the milk of lactating BLG-transgenic mice. These authors calculated that the concentration of ovine BLG in the milk of line 45 mice was between 14-22mgs/ml.

Melamed and Friedman (1993) [269] found that tolerance to 20mgs of OVA occurred quickly in adult mice following a single intragastric feed. However tolerance was short lived (21 days) in the absence of additional antigen. "BLG-suckled" mice were exposed continuously to antigen until immunisation since they were weaned onto a diet containing 1.25% bovine BLG after suckling (resulting in ingestion of an estimated 36.25mgs of BLG/day). Following immunisation these mice were as responsive to ovine BLG as a control group fed on a BLG free diet. Continuous BLG exposure following weaning is therefore unlikely to be the cause of responsiveness observed in this study.

Hanson (1981) [374] suggested that the effects of oral tolerance were best seen if mice were immunised 2 rather than 6 weeks following feeding of antigen, thus suggesting that the tolerogenic effect of feeding diminished with time. Mice in my study were immunised 9 weeks after the last oral administration of antigen and it remains a possibility that hyporesponsiveness to ovine BLG had declined over this period inspite of the presence of BLG in the diet.

In summary, although no oral tolerance towards ovine BLG was evident in adult non-transgenic and BLG-transgenic mice suckled on BLG-containing milk, (during the first 21 days of

life) oral tolerance to bovine BLG could be achieved in adult CBA/Ca mice. It is possible that oral tolerance may have occurred following suckling on BLG-containing milk for a short period but had waned over the length of time between weaning and challenge. The question of whether oral tolerance to maternal milk proteins normally occurs in the suckling offspring was not analysed in this study.

Oral tolerance to bovine BLG did occur in adult CBA/Ca mice fed this protein in their drinking water. However the level of suppression of both the antibody and T cell response in this study was not as impressive as those described by Enomoto et al (1993) [331]. Various differences including experimental procedure, the strain of mouse used and the amount of bovine BLG fed could explain the differences seen between these experiments. Of these three differences the first two may be of most importance.

Oral tolerance to OVA has been shown to be affected by mouse genetic backgrounds [266, 267]. For example BALB/c (H-2^d) mice fed OVA had a 85-90% suppressed OVA specific DTH response whilst BALB/b (H-2^b) mice also fed this protein were responsive to OVA at a DTH level. In contrast (BALB/c X BALB/b) F1 mice fed OVA had a 63% suppressed OVA specific DTH response. The relatively low levels of oral tolerance to bovine BLG that occurred in the CBA/Ca mouse (H-2^k), used in my study, may be a consequence of MHC genotype: the BALB/c mice used in Enomoto's study were (H-2^d). Although the susceptibility of other mouse strains to oral tolerance to BLG was not carried out in my study, Enomoto et al (1993) [331] found that both C3H/He (H-2^k) and C57BL/6 (H-2^b) mice were susceptible to oral tolerance to whey proteins and that the levels of suppression in these mice was similar to that of BALB/c mice. Although CBA/Ca and C3H/He mice both express H-2^k it was also shown by Lamont et al (1988) [267] that H-2^k mice show marked variation in the induction of oral tolerance to OVA suggesting an additional role of genetic factors outwith the MHC in oral tolerance.

Enomoto et al (1993) [331] calculated that mice received 0.29gs of bovine BLG per day in their study. In contrast, mice in my study received 25-86mgs of bovine BLG depending on the presence or absence of whey protein in the solid food diet. For example a diet containing 1.25% of BLG resulted in the ingestion of 36.25mgs of BLG (based on an average daily intake of 2.9gs). The difference between Enomoto's results and my result's could be affected by the amount of BLG ingested, the greater the amount the more tolerance.

Ingestion of a small amount of BLG may result in no, or very little, protein reaching the systemic system due to denaturation in the stomach, whilst ingestion of large amounts of undigested BLG may result in large amounts of protein reaching the systemic blood supply. This hypothesis is however unlikely to be relevant since, 1) BLG is stable at pH 3.5, the pH of the stomach, [303] thus allowing undigested BLG, regardless of amount ingested to reach the systemic blood supply, 2) denatured whey protein can induce oral tolerance to BLG [331], although in this study large quantities of denatured whey protein (560mgs/day) were given which may have allowed a large quantity of protein to reach the systemic circulation and c) the amount of BLG fed in my study was the same or greater than the amount of OVA ingested in some experiments [261, 266, 267, 288, 378, 379]. Hence there should have been sufficient antigen to induce tolerance.

Oral tolerance, at both the T cell and antibody level, to another milk protein has been documented [330, 332-334]. Both anti-casein antibody and T cell responses were suppressed (87.8%) in mice fed a solid food diet in which casein was the only protein. Although these mice were tolerant a weak but significant responses was observed and it was found that tolerance in this case was to the immunodominant peptides of casein not to the cryptic epitopes. It is unlikely that the relatively limited tolerance to bovine BLG in my study was an immunodominance phenomenon since suppression of B and T cell responses to bovine BLG was almost 100% in the Enomoto study.

As described in Chapter 1 oral tolerance occurs either via suppression or anergy. In this study the mechanism of oral tolerance was not analysed. Anergy is thought to occur when soluble antigen, in the systemic system, is presented to T cells in the absence of a co-stimulatory signal. Soluble bovine BLG has been found in the systemic blood supply of mice fed a whey protein containing solid food diet [331] suggesting that oral tolerance to this protein maybe via anergy.

8.8) Summary of future work

i) T cell tolerance.

T cell tolerance should be further investigated using haptenated BLG compounds. If T cell tolerance exists in BLG-transgenic mice then no anti-hapten responses would occur and this would facilitate investigations into the T cell subset(s) affected. As suggested earlier T_H2 cells rather than T_H1 cells maybe tolerant and this hypothesis should be further investigated by the analysis of both cytokine and antibody profiles of BLG-transgenics versus non-transgenic mice following immunisation with BLG plus Alum or FCA.

The mechanism of T cell tolerance could also be analysed. Although no BLG mRNA expression was found in various tissues, including the thymus following Northern blotting, future work should re-investigate expression using a technique known to be sensitive for small amounts of RNA, RT-PCR.

In conjunction with the forementioned experiments thymic involvement should also be analysed using thymic chimaeras, for example, grafting BLG-transgenic and non-transgenic thymi under the kidney capsule of CBA/Ca nude mice such that host derived T cells develop in donor thymi. BLG expression in the transgenic thymi would be expected to induce tolerance in these mice.

ii) OVA transgenic mice

It would be interesting to repeat the experiments detailed in this thesis using an OVA transgenic system. This would

involve the production of transgenic mice with OVA under the control of BLG promoter elements. Much is known about the immune response to OVA making it the ideal antigen for such a study.

iii) B cell tolerance

B cell tolerance should be analysed further through the use of hapten-carrier experiments, for example coupling BLG (hapten) to SRBC (carrier). Since B cell tolerance can co-exist with T cell tolerance the above situation bypasses T cell tolerance by providing an alternative T cell help (SRBC specific cells) to hapten specific B cells. If BLG specific B cells are tolerant in BLG-transgenic mice it could be predicted that no anti-BLG antibody response would be detected even in the presence of T cell help.

If B cell tolerance is evident the mechanism involved could be investigated. At present the favoured mechanism is deletion of BLG specific B cells within the bone marrow although the presence of BLG in this tissue has not been evaluated. Future experiments should therefore investigate whether BLG mRNA or protein is present in the marrow stroma. Although B cell anergy has been ruled out in this model since soluble BLG is not found in these mice, anergy could be further investigated via the transfer of transgenic B cells into a non-transgenic host. If tolerance was found to be transferable anergy may also be involved.

iv) BLG as an oral tolerogen

Oral tolerance to BLG did not occur in mice suckled on BLG-containing milk as compared to mice suckled on "normal" mouse milk. The lack of tolerance was attributed firstly to the lack of a continuous source of tolerogen and secondly to the fact that the mice were challenged 3 months from birth by which time oral tolerance may have subsided. Future experiments should therefore investigate whether tolerance to BLG did occur but had waned through time. These experiments

would involve challenging BLG-suckled mice at various time points after weaning and before mice reach 3 months of age.
It would also be of interest to test whether tolerance to other murine milk proteins occurred in suckling offspring.
Adult mice given bovine BLG in their drinking water were tolerant to this protein although the level of tolerance was not comparable to that found in other laboratories [331]. One reason for this was that CBA/Ca mice were tested in my study whilst BALB/c mice were tested in Enomoto's study. It would be interesting to test the susceptibiliy of BALB/c mice to oral tolerance induction with bovine BLG.
It is possible that BLG is not a good protein to study with respect to oral tolerance. The production of transgenic mice producing OVA in milk, during lactation, would provide a better model for the study of induction of oral tolerance via suckling, since much data is available on the mechanisms of oral tolerance to this protein.
In future experiments the mechanism of BLG oral tolerance should be analysed. In vitro anergy should be investigated by analysis of cytokine production. In vivo the mechanism could be investigated by dissection of the IgG subclass response since oral tolerance induced through anergy results in the selective tolerisation of T_H1 cells (resulting in decreased IgG2a antibody levels) but not T_H2 cell [270, 273, 280]. In the present study only the total IgG response was analysed.

1. Banchereau, J. and F. Rousset, *Human B lymphocytes: phenotype, proliferation and differentiation.* Adv Immunol, 1992. 52: p. 125-262.

2. Reth, M., *Regulation of B-cell development by pre-B-cell receptors.* Developmental Immunology, 1991. 1: p. 198-199.

3. Melcher, F., *et al., The surrogate light chain in B-cell development.* Immunol Today, 1993. 14: p. 60-68.

4. Kehrl, J.H., *et al., Molecular mechanisms regulating CD19, CD20 and CD22 gene expression.* Immunol Today, 1994. 15: p. 432-436.

5. Pleiman, C.M., D. D'Ambrosio, and J.C. Cambier, *The B-cell antigen receptor complex: structure and signal transduction.* Immunol Today, 1994. 15: p. 393-405.

6. Chen, C., et al *Probing immune functions in RAG-deficient mice.* Curr Opin Immunol, 1994. 6: p. 313-319.

7. Tarlinton, D., *B-cell differentiation in the bone marrow and the periphery.* Immunol Rev, 1994. 137: p. 203-229.

8. Rolink, A., *et al., Two pathways of B-lymphocyte development in mouse bone marrow and the roles of surrogate L chain in this development.* Immunol Rev, 1994. 137: p. 185-203.

9. Loffert, D., *et al., Early B-cell development in the mouse:Insight from mutations introduced by gene targeting.* Immunol Rev, 1994. 137: p. 135-155.

10. Grandien, A., A. Coutinho, and J. Anderson, *Selective peripheral expansion and activation of B cells expressing endogenous immunoglobulin on μ-transgenic mice.* Eur J Immunol, 1990. 20: p. 991-998.

11. Cumano, A., *et al., Development of B lymphocytes from lymphoid committed and uncommitted progenitors.* Immunol Rev, 1994. 137: p. 5-35.

12. Liu, Y.-J., *et al., Germinal centres in T-cell-dependent antibody responses.* Immunol Today, 1992. 13: p. 17-21.

13. Berek, C. and M. Zeigner, *The maturation of the immune response.* Immunol Today, 1993. 14: p. 400-404.

14. Nunez, G., *et al., Bcl-2 and Bcl-x: regulatory switches for lymphoid death and survival.* Immunol Today, 1994. 15: p. 582-588.

15. Hagman, J. and R. Grosschedl, *Regulation of gene expression at early stages of B-cell differentiation.* Curr Opin Immunol, 1994. 6: p. 222-230.

16. Law, C.-E. and E.A. Clark, *Cell-cell interactions that regulate the development of B-lineage cells.* Curr Opin Immunol, 1994. 6: p. 238-247.

17. Rajewski, K., *Early and late B-cell development in the mouse.* Curr Opin Immunol, 1992. 4: p. 171-176.

18. Kincade, P.W. and J.M. Gimble, *B lymphocytes,* in *Fundamental Immunology,* W.E. Paul, Editor. 1993, Raven Press, Ltd: New York. p. 43-68.

19. Bretscher, P. and M. Cohn, *A theory of "self-non-self" discrimination: analysis and induction involve the recognition of one or two determinants as an antigen, respectively.* Science, 1970. 169: p. 1042-1049.

20. Paraham, P., *Intolerable secretion in tolerant transgenic mice.* Nature, 1988. 333: p. 500-503.

21. Berek, C. and C. Milstein, *The dynamic nature of the antibody repertoire.* Immunol Rev, 1988. 105: p. 5-26.

22. Diamond, B. and M.D. Scharff, *Somatic mutations of the T-15 heavy chain gives rise to an antibody with an autoantibody specificity.* Proc Natl Acad Sci USA, 1984. 81: p. 5841-5844.

23. Basten, A., *et al., Self tolerance in the B cell repertoire.* Immunol Rev, 1987. 122: p. 5-19.

24. Nemazee, D. and K. Buerki, *Clonal deletion of autoreactive B lymphocytes in bone marrow chimaeras.* Proc Natl Acad Sci USA, 1989. 86: p. 8039-8043.

25. Nemazee, D.A. and K. Burki, *Clonal deletion of B lymphocytes in transgenic mouse bearing anti-MHC class I antibody genes.* Nature, 1989. 337: p. 562-566.

26. Chen, C., *et al.*, *Deletion and editing of B cell that express antibodies to DNA.* J Immunol, 1994. 152: p. 1970-1982.

27. Muller, W., *et al.*, *Membrane-bound IgM obstructs B cell development in transgenic mice.* Eur J Immunol, 1989. 19: p. 923-928.

28. Forni, L., *Extensive splenic B cell activation in IgM-Transgenic mice.* Eur J Immunol, 1990. 20: p. 983-989.

29. Lamers, M.C., *et al.*, *Immune status of a μ, κ transgenic mouse line. Deficient response to bacterially related antigens.* Eur J Immunol, 1989. 19: p. 459-468.

30. Stall, A.M., *et al.*, *Rearrangement and expression of endogenous immunoglobulin genes occur in many murine B cells expressing transgenic membrane IgM.* Proc Natl Acad Sci USA, 1988. 85: p. 3546-3550.

31. Radic, M.Z., *et al.*, *B lymphocytes may escape tolerance by revising their antigen receptors.* J Exp Med, 1993. 177: p. 1165-1173.

32. Tiegs, S.L., D.M. Russell, and D. Nemazee, *Receptor editing in self-reactive bone marrow B cells.* J Exp Med, 1993. 177: p. 1009-1020.

33. Tighe, H., *et al.*, *Function of B cells expressing a human immunoglobulin M rheumatoid factor autoantibody in transgenic mice.* J Exp Med, 1993. 177: p. 109-118.

34. Gu, H., *Gene targeting and its application to the study of B-cell development.* Curr Opin Immunol, 1994. 6: p. 308-312.

35. Gay, D., *et al.*, *Receptor editing: an approach by autoreactive B cells to escape tolerance.* J Exp Med, 1993. 177: p. 999-1008.

36. Rubio, C.F. and D. Nemazee, *B cell tolerance in autoimmune prone mice.* Arthritis Rheum Suppl 34, 1991. 107: p. S50.

37. Hartley, S.B., *et al.*, *Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens.* Nature, 1991. 353: p. 765-769.

38. Hartley, S.B., *et al.*, *Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death.* Cell, 1993. 72: p. 325-335.

39. Kamesaki, H., *et al.*, *Role of bcl-2 and IL-5 regulation of anti-IgM-induced growth arrest and apoptosis in immature B cell lines. A cooperative regulation model for B cell clonal deletion.* J Immunol, 1994. 152: p. 3294-3305.

40. Neuberger, M.S., *Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells.* EMBO J, 1983. 2: p. 1373-1378.

41. Okamoto, M., *et al.*, *A transgenic model of autoimmune hemolytic anaemia.* J Exp Med, 1992. 175: p. 71-79.

42. Goodnow, C.C., *Safe havens for self-reactive cells.* Autoimmunity, 1992. 2: p. 417-419.

43. Brombacher, F., G. Kohler, and H. Eibel, *B cell tolerance in mice transgenic for anti-CD8 immunoglobulin μ chain.* J Exp Med, 1991. 174: p. 1335-1346.

44. Nitschke, D., *et al.*, *Immunoglobulin D-deficient mice can mount normal immune responses to thymus-independent and -dependent antigens.* Proc Natl Acad Sci USA, 1993. 90: p. 1887-1891.

45. Carsetti, R., G. Kohler, and M.C. Lamers, *A role for immunoglobulin D: interference with tolerance induction.* Eur J Immunol, 1993. 23: p. 168-178.

46. Erikson, J., *et al., Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice.* Nature, 1991. 349: p. 331-334.

47. Goodnow, C.C., *et al., Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice.* Nature, 1988. 334: p. 674-682.

48. Mason, D.Y., M. Jones, and C.C. Goodnow, *Developmental and follicular localisation of tolerant B lymphocytes in lysozyme/anti-lysozyme IgM/IgD transgenic mice.* Int Immunol, 1992. 4: p. 163-175.

49. Goodnow, C.C., *et al., Induction of self-tolerance in mature peripheral B lymphocytes.* Nature, 1989. 342: p. 385-390.

50. Bell, S.E. and C.C. Goodnow, *A selective defect in IgM antigen receptor synthesis and transport causes loss of cell surface IgM expression on tolerant B lymphocytes.* EMBO J, 1994. 13: p. 816-826.

51. Fulcher, D.A. and A. Basten, *Reduced life span of anergic self-reactive B cells in a double-transgenic model.* J Exp Med, 1994. 179: p. 125-134.

52. Adelstein, S., *et al., Induction of self-tolerance in T cells but not B cells of transgenic mice expressing little self antigen.* Science, 1991. 251: p. 1223-1225.

53. Adams, E., A. Basten, and C.C. Goodnow, *Intrinsic B-cell hyporesponsiveness accounts for self-tolerance in lysozyme/anti-lysozyme double-transgenic mice.* Proc Natl Acad Sci USA, 1990. 87: p. 5687-5691.

54. Goodnow, C.C., R. Brink, and E. Adams, *Breakdown of self-tolerance in anergic B lymphocyes.* Nature, 1991. 352: p. 532-536.

55. Eris, J.M., *et al., Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-signals but are defective in antigen-receptor-mediated functions.* Proc Natl Acad Sci USA, 1994. 91: p. 4392-4396.

56. Kanost, D. and J. McCluskey, *Anergic B cells constitutively present self antigen: enhanced immunoglobulin receptor-mediated presentation of antigenic determinants by B cells is hierarchical.* Eur J Immunol, 1994. 24: p. 1186-1193.

57. Cooke, M.P., *et al., Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells.* J Exp Med, 1994. 179: p. 425-438.

58. Tsao, B.P., *et al., B cells are anergic in transgenic mice that express IgM anti-DNA antibodies.* Eur J Immunol, 1993. 23: p. 2332-2339.

59. Offen, D., *et al., Induction of tolerance to an IgG autoantibody.* Proc Natl Acad Sci, 1992. 89: p. 8332-8336.

60. Steinhoff, U., *et al., Virus or a hapten-carrier complex can activate autoreactive B cell by providing linked T help.* Eur J Immunol, 1994. 24: p. 773-776.

61. Bachmann, M.F., *et al., The influence of antigen organisation on B cell responsiveness.* Science, 1993. 262: p. 1448-1451.

62. Savino, W., D.M.S. Villa-Verde, and J. Lannes-Vieira, *Extracellular matrix proteins in intrathymic T-cell migration and differentiation.* Immunol Today, 1993. 14: p. 158-164.

63. van Ewijk, W., *T-cell differentiation is influenced by thymic microenvironments.* Annu Rev Immunol, 1991. 9: p. 591-615.

64. Ritter, M.A. and R.L. Boyd, *Development in the thymus: it takes two to tango.* Immunol Today, 1993. 14: p. 462-469.

65. Ritter, M.A. and I.N. Crispe, *The thymus*. In Focus, ed. D. Male. 1992, New York: Oxford University Press.

66. von Boehmer, H., *Thymic selection: a matter of life and death.* Immunol Today, 1992. 13: p. 454-459.

67. Boyd, R.L., *et al., The thymic microenvironment.* Immunol Today, 1993. 14: p. 445-458.

68. Kisielow, P. and H. von Boehmer, *Development and selection of T cells: Facts and Puzzles.* Adv Immunol, 1995. 58: p. 87-209.

69. Weidmeier, S.E., *et al.*, *Effect of ionizing radiation on thymic epithelial cell function I. Radiation-spared thymic epithelial grafts expedite the recovery of T cell function in lethally irradiated and fetal liver reconstituted mice.* J Immunol, 1988. 140: p. 21-29.

70. Huiskamp, R., E. van Vliet, and W. Van Ewijk, *Repopulation of the mouse thymus after sublethal fission neutron irradiation II. Sequential changes in the thymic microenvironment.* J Immunol, 1985. 134: p. 2170-2178.

71. Low, T.L.K. and A.L. Goldstein, *The chemistry and biology of thymosin II. Amino acid sequence analysis of thymosin α_1 and polypeptide β_1.* J Biol Chem, 1979. 254: p. 987-995.

72. Low, T.L.K., *et al.*, *The chemistry and biology of thymosin I.Isolation, charaterisation and biological activities of thymosin α_1 and polypeptide β_1 from calf thymus.* J Biol Chem, 1979. 254: p. 981-986.

73. Dargemont, C., *et al.*, *Thymotaxin, a chemotactic protein, is identical to β2-microglobulin.* Science, 1989. 246: p. 803-806.

74. Kyewski, B.A., *Thymic nurse cells: possible sites of T-cell selection.* Immunol Today, 1986. 7: p. 374-379.

75. Wekerle, H. and U.-P. Ketelsen, *Thymic-nurse cells-Ia-bearing epithelium involved in T-lymphocyte differentiation?* Nature, 1980. 283: p. 402-404.

76. Rieker, T., *et al.*, *In situ analysis of in ovo graft-vs-host reactiion induced by thymic nurse cell lymphocytes.* Eur J Immunol, 1993. 23: p. 904-910.

77. Philip, D., *et al.*, *The binding, internalisation, and release of thymocytes by thymic nurse cells.* Cell Immunol, 1993. 148: p. 301-315.

78. Gao, X., *et al.*, *Thymic nurse cell clone supports the differentiation of CD4-8- thymocytes into CD4+8+ thymocytes in vitro.* Immunol Lett, 1993. 35: p. 169-176.

79. Nishimura, T., *et al.*, *Thymic stromal cell clone with nursing activity supports the growth and differentiation of murine CD4+8+ thymocytes in vitro.* J Immunol, 1990. 145: p. 4012-4017.

80. Ardavin, C., *et al.*, *Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population.* Nature, 1993. 362: p. 761-763.

81. Mombaerts, P., *et al.*, *Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages.* Nature, 1992. 360: p. 225-231.

82. Mallick, C.A., *et al.*, *Rearrangement and diversity of T cell receptor β chain genes in thymocytes: a critical role for the β chain in development.* Cell, 1993. 73: p. 513-519.

83. Palmer, D.B., A. Hayday, and M.J. Owen, *Is TCRβ expression an essential event in early thymocyte development?.* Immunol Today, 1993. 14: p. 460-462.

84. Shores, E.W., *et al.*, *T cell receptor-negative thymocytes from SCID mice can be induced to enter the CD4/CD8 differentiation pathway.* Eur J Immunol, 1990. 20: p. 69-77.

85. Scollay, R., W.F. Chen, and K. Shortman, *The functional capabilities of cells leaving the thymus.* J Immunol, 1984. 132: p. 25-30.

86. Husmann, L.A., *et al.*, *Thymocyte subpopulations during early fetal development in the BALB/c mouse.* J Immunol, 1988. 141: p. 736-740.

87. van Meerwijk, J.P.M., *et al.*, *Allelic exclusion at DNA rearrangement level is required to prevent co-expression of two distinct T cell receptor β genes.* J Exp Med, 1991. 174: p. 815-819.

88. Krimpenfort, P., *et al., T cell depletion in transgenic mice carrying a mutant gene for TCR-β.* Nature, 1989. 341: p. 742-746.

89. Benoist, C. and D. Mathis, *Generation of the αβ T-cell repertoire.* Curr Opin Immunol, 1992. 4: p. 156-161.

90. Philpott, K.L., *et al., Lymphoid development in mice congenitally lacking T cell receptor αβ-expressing cells.* Science, 1992. 256: p. 1448-1452.

91. Snodgrass, H.R., *et al., Expression of T-cell antigen receptor genes during fetal development in the thymus.* Nature, 1985. 315: p. 232-233.

92. Raulet, D.H., *et al., Developmental regulation of T-cell receptor gene expression.* Nature, 1985. 314: p. 103-107.

93. Bevan, M.J., *In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells.* Nature, 1977. 269: p. 417-418.

94. Sha, W.C., *et al., Positive and negative selection of an antigen receptor on T cells in transgenic mice.* Nature, 1988. 336: p. 73-76.

95. Teh, H.S., *et al., Thymic major histocompatibility complex antigens and the αβ T-cell receptor determine the CD4/CD8 phenotype of T cells.* Nature, 1988. 335: p. 229-233.

96. Kay, R.A. and A. Ferguson, *The immunological consequences of feeding cholera toxin II. Mechanisms responsible for the induction of oral tolerance for DTH.* Immunology, 1989. 66: p. 416-421.

97. Kisielow, P., *et al., Positive selection of antigen-specific T cells in thymus by restricting MHC molecules.* Nature, 1988. 335: p. 730-733.

98. Scott, B., *et al., The generation of mature T cells requires interaction of the αβ T-cell receptor with major histocompatibility antigens.* Nature, 1989. 338: p. 591-593.

99. Benoist, C. and D. Mathis, *Positive selection of the T cell repertoire: Where and when does it occur?* Cell, 1989. 58: p. 1027-1033.

100. Berg, L.J., *et al.*, *Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand.* Cell, 1989. 58: p. 1035-1046.

101. Berg, L.J., G.D. Frank, and M.M. Davis, *The effects of MHC gene dosage and allelic variation on T cell receptor selection.* Cell, 1990. 60: p. 1043-1053.

102. Ohashi, P.S., *et al.*, *Distinct sequence of negative or positive selection implied by thymocyte T-cell receptor densities.* Nature, 1990. 346: p. 861-863.

103. Kaye, J., *et al.*, *Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor.* Nature, 1989. 341: p. 746-749.

104. Berg, L.J., *et al.*, *Phenotypic differences between α/β versus β T-cell receptor transgenic mice undergoing negative selection.* Nature, 1989. 340: p. 559-562.

105. Anderson, G., *et al.*, *MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus.* Nature, 1993. 362: p. 70-73.

106. Hugo, P., *et al.*, *A cell line that can induce thymocyte positive selection.* Nature, 1992. 362: p. 679-682.

107. Vukmanovic, S., *et al.*, *Positive selection of T-lymphocytes induced by intrathymic injection of a thymic epithelial cell line.* Nature, 1992. 359: p. 729-732.

108. Bix, M. and D. Raulet, *Inefficient positive selection of T cells directed by haematopoietic cells.* Nature, 1992. 359: p. 330-333.

109. Pawlowski, T., *et al.*, *Positive selection of T lymphocytes on fibroblasts.* Nature, 1993. 364: p. 642-645.

110. Koller, B.H., *et al., Normal development of mice deficient in β_2M, MHC class I proteins, and CD8+ T cells.* Science, 1990. 248: p. 1227-12230.

111. Zijlstra, M., *et al., β2-Microglobulin deficient mice lack CD4-8+ cytolytic T cells.* Nature, 1990. 344: p. 742-746.

112. Van Kaer, L., *et al., TAP-1 mutant mice are deficient in antigen presentation, surface Class 1 molecules, and CD4-8+ T cells.* Cell, 1992. 71: p. 1205-1214.

113. Cosgrove, D., *et al., Mice lacking MHC class II molecules.* Cell, 1991. 66: p. 1051-1066.

114. Grusby, M.J., *et al., Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice.* Science, 1991. 253: p. 1417-1420.

115. Viville, S., *et al., Mice lacking the MHC class II-associated invariant chain.* Cell, 1993. 72: p. 635-648.

116. Aldrich, C.J., *et al., Negative and positive selection of antigen-specific cytotoxic T lymphocytes affected by the α3 domain of MHC I molecules.* Nature, 1991. 352: p. 718-720.

117. Ashton-Rickardt, P.G., *et al., Repertoire-determining role of peptide in the positive selection of CD8+ T cells.* Immunol Rev, 1993. 135: p. 157-183.

118. Falk, K., *et al., Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules.* Nature, 1991. 351: p. 290-296.

119. Hunt, D.F., *et al., Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry.* Science, 1992. 259: p. 1261-1263.

120. Nikolic-Zugic, J. and M.J. Bevan, *Role of self-peptides in positively selecting the T-cell repertoire.* Nature, 1990. 344: p. 65-67.

121. Ohashi, P.S., *et al., Enhanced positive selection of a transgenic TCR by a restricted element that does not permit negative selection.* Int Immunol, 1993. 5: p. 131-138.

122. Sha, W.C., *et al., Positive selection of transgenic receptor-bearing thymocytes by K^b antigen is altered by K^b mutations that involve peptide binding.* Proc Natl Acad Sci USA, 1990. 87: p. 6186-6190.

123. Jacobs, H., *et al., Mutations in the major histocompatibility complex Class 1 antigen presenting groove affect both negative and positive selection of T cells.* Eur J Immunol, 1990. 20: p. 2333-2337.

124. Vukmanovic, S., M.J. Bevan, and K.A. Hogquist, *The specificity of positive selection: MHC and peptides.* Immunol Rev, 1993. 135: p. 51-66.

125. Rahemtulla, A., *et al., Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4.* Nature, 1991. 353: p. 180-184.

126. Seong, R.H., J.W. Chamberlain, and J.R. Parnes, *Signal for T-cell differentiation to a CD4 cell lineage is delivered by CD4 transmembrane region and/or cytoplasmic tail.* Nature, 1992. 356: p. 718-720.

127. Fung-Leung, W.-P., *et al., CD8 is needed for development of cytotoxic T cells and not helper T cells.* Cell, 1991. 65: p. 443-449.

128. Fung-Leung, W.-P., *et al., CD8 is needed for positive selection but differentially required for negative selection of T cells during thymic ontogeny.* Eur J Immunol, 1993. 23: p. 212-216.

129. Schilham, M.W., *et al., Alloreactive cytotoxic T cells can develop and function in mice lacking both CD4 and CD8.* Eur J Immunol, 1993. 23: p. 1299-1304.

130. Borgulya, P., *et al., Development of CD4 and CD8 lineage of T cells: instructive versus selection.* EMBO J, 1991. 10: p. 913-918.

131. Robey, E.A., *et al., Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage.* Cell, 1991. 64: p. 99-107.

132. Swat, W., *et al.*, *Phenotypic changes accompanying positive selection of CD4+CD8+ thymocytes.* Eur J Immunol, 1992. 22: p. 2367-2372.

133. Swat, W., *et al.*, *CD69 expression during selection and maturation of CD4+8+ thymocytes.* Eur J Immunol, 1993. 23: p. 739-746.

134. Chan, S.H., *et al.*, *Another view of the selective model of thymocyte selection.* Cell, 1993. 73: p. 225-236.

135. Davis, C.B., *et al.*, *Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes.* Cell, 1993. 73: p. 237-247.

136. Teh, H.-S., *et al.*, *Deletion of autospecific T cells in T cell receptor (TCR) transgenic mice spares cells with normal TCR levels and low levels of CD8 molecules.* J Exp Med, 1989. 169: p. 795-806.

137. Cabaniols, J.-P., *et al.*, *Dose-dependent T cell tolerance to an immunodominant self peptide.* Eur J Immunol, 1994. 24: p. 1743-1749.

138. Pircher, H., *et al.*, *Tolerance induction by clonal deletion of CD4+8+ thymocytes in vitro does not require dedicated antigen-presenting cells.* Eur J Immunol, 1993. 23: p. 669-674.

139. Carlow, D.A., *et al.*, *Peripheral tolerance through clonal deletion of mature CD4-CD8+ T cells.* Int Immunol, 1992. 4: p. 599-610.

140. Tomonari, K. and S. Fairchild, *Positive and negative selection of Tcrb-V6+ T cells.* Immunogenetics, 1992. 36: p. 230-237.

141. Kappler, J.W., *et al.*, *Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex.* Nature, 1988. 332: p. 35-40.

142. MacDonald, H.R., *et al.*, *T-cell receptor Vβ usage predicts reactivity and tolerance to Mls^a-encoded antigens.* Nature, 1988. 332: p. 40-45.

143. Webb, S.R. and J. Sprent, *Induction of neonatal tolerance to Mls^a antigens by CD8+ T cells.* Science, 1990. 248: p. 1643-1646.

144. Fry, A.M. and L.A. Matis, *Self-tolerance alters T-cell receptor expression in an antigen-specific MHC restricted immune response.* Nature, 1988. 335: p. 830-832.

145. Fry, A.M., *et al.*, *Thymic requirement for clonal deletion during T cell development.* Science, 1989. 246: p. 1044-1046.

146. Woodland, D.L., *et al.*, *An endogenous retrovirus mediating deletion of $\alpha\beta$ T cells?* Nature, 1991. 349: p. 529-530.

147. Kappler, J.W., N. Roehmer, and P. Marrack, *T cell tolerance by clonal elimination in the thymus.* Cell, 1987. 49: p. 273-280.

148. Bluthmann, H., *et al.*, *T-cell-specific deletion of T-cell receptor transgene allows functional rearrangement of endogenous α - and β-genes.* Nature, 1988. 334: p. 156-159.

149. Blackman, M., J. Kappler, and P. Marrack, *The role of the T cell receptor in positive and negative of developing T cells.* Science, 1990. 248: p. 1335-1341.

150. Kisielow, P., *et al.*, *Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes.* Nature, 1988. 333: p. 742-746.

151. Yui, K., *et al.*, *Self-reactive T cells can escape clonal deletion in T-cell receptor Vβ8.1 transgenic mice.* Proc Natl Acad Sci USA, 1990. 87: p. 7135-7139.

152. Pircher, H., *et al.*, *Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen.* Nature, 1989. 342: p. 559-561.

153. Van Ewijk, W., *et al.*, *Compartmentalization of MHC Class II gene expression in transgenic mice.* Cell, 1988. 53: p. 357-370.

154. Pircher, H., *et al., T cell tolerance to Mls^a encoded antigens in T cell receptor Vβ8.1 chain transgenic mice.* EMBO J, 1989. 8: p. 719-727.

155. Fowlkes, B.J., R.H. Schwartz, and D.M. Pardoll, *Deletion of self-reactive thymocytes occurs at a CD4+8+ precursor stage.* Nature, 1988. 334: p. 620-623.

156. Blackman, M.A., *et al., A role for clonal inactivation in T cell tolerance to Mls-1^a.* Nature, 1990. 345: p. 540-542.

157. Vasquez, N.J., J. Kaye, and S.M. Hedrick, *In vivo and in vitro clonal deletion of double-positive thymocytes.* J Exp Med, 1992. 175: p. 1307-1316.

158. Swat, W., *et al., Clonal deletion of immature CD4+8+ thymocytes in suspension cultures by extrathymic antigen-presenting cells.* Nature, 1991. 351: p. 150-153.

159. Spain, L.M. and L.J. Berg, *Developmental regulation of thymocyte susceptibility to deletion by "self" peptide.* J Exp Med, 1992. 176: p. 213-223.

160. Takahama, Y., E.W. Shores, and A. Singer, *Negative selection of precursor thymocytes before their differentiation into CD4+CD8+ cells.* Science, 1992. 258: p. 653-656.

161. Swat, W., H. von Boehmer, and P. Kisielow, *Central tolerance: clonal deletion or clonal arrest?* Eur J Immunol, 1994. 24: p. 485-487.

162. Zal, T., A. Volkmann, and B. Stockinger, *Mechanisms of tolerance induction in major histocompatibility complex Class II-restricted T cells specific for a blood-borne self-antigen.* J Exp Med, 1994. 180: p. 2089-2099.

163. Stockinger, B. and B. Hausmann, *Functional recognition of in vivo processed self antigen.* Int Immunol, 1994. 6: p. 247-254.

164. Robey, E.A., *et al., The level of CD8 expression can determine the outcome of thymic selection.* Cell, 1992. 69: p. 1089-1096.

165. Homer, R.J., *et al.*, *T cell unresponsiveness correlates with quantitative TCR levels in a transgenic model.* Int Immunol, 1993. 5: p. 1495-1500.

166. Lee, N.A., D.Y. Loh, and E. Lacy, *CD8 surface levels alter the fate of α/β T cell receptor-expressing thymocytes in transgenic mice.* J Exp Med, 1992. 175: p. 1013-1023.

167. Sherman, L.A., *et al.*, *Selecting T cell receptors with high affinity for self-MHC by decreasing the contribution of CD8.* Science, 1992. 258: p. 815-818.

168. Hsu, B.L., B.D. Evavold, and P.M. Allen, *Modulation of T cell development by an endogenous altered peptide ligand.* J Exp Med, 1995. 181: p. 805-810.

169. Sebzda, E., *et al.*, *Positive and negative thymocyte selection induced by different concentrations of a single peptide.* Science, 1994. 263: p. 1615-1618.

170. Oehen, S.U., *et al.*, *Escape of thymocytes and mature T cells from clonal deletion due to limiting tolerogen expression levels.* Cell Immunol, 1994. 158: p. 342-352.

171. Knobloch, M., *et al.*, *T cell activation and thymic tolerance induction require different adhesion intensities of the CD8 co-receptor.* Int Immunol, 1992. 4: p. 1169-1174.

172. Ingold, A.L., *et al.*, *Co-engagenent of CD8 with the T cell receptor is required for negative selection.* Nature, 1991. 352: p. 721-723.

173. MacDonald, H.R., H. Hengartner, and T. Pedrazzini, *Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment.* Nature, 1988. 335: p. 174-176.

174. Wallace, V.A., *et al.*, *CD4 expression is differentially required for deletion of Mls-1^a reactive T cells.* J Exp Med, 1992. 176: p. 1459-1463.

175. Nakayama, K.-I. and D.Y. Loh, *No requirement for $p56^{lck}$ in the antigen-stimulated clonal deletion of thymocytes.* Science, 1992. 257: p. 94-96.

176. Sponaas, A.-M., *et al.*, *Induction of tolerance to self MHC class 1 molecules expressed under the control of the milk protein or β-globin gene promoters.* Int Immunol, 1994. 6: p. 277-287.

177. Ramsdell, F., T. Lantz, and B.J. Fowlkes, *A nondeletional mechanism of thymic self tolerance.* Science, 1989. 246: p. 1038-1041.

178. Pircher, H., *et al.*, *Thymocytes can tolerize thymocytes by clonal deletion in vitro.* Int Immunol, 1992. 4: p. 1065-1069.

179. Simpson, S.J., P. Tomlinson, and A.L. Mellor, *Immunological consequences of T cell-specific expression of H-2K^b molecules in transgenic mice.* Int Immunol, 1993. 5: p. 189-198.

180. Iwabuchi, K., *et al.*, *Cellular and peptide requirements for in vitro clonal deletion of immature thymocytes.* Proc Natl Acad Sci USA, 1992. 89: p. 9000-9004.

181. Mazda, O., *et al.*, *Requirement of dendritic cells and B cells in the clonal deletion of Mls-reactive T cells in the thymus.* J Exp Med, 1991. 173: p. 539-547.

182. Carlow, D.A., *et al.*, *Deletion of antigen-specific immature thymocytes by dendritic cells requires LFA-1/ICAM interaction.* J Immunol, 1992. 148: p. 1595-1603.

183. Miyazaki, T., G. Suzuki, and K.-I. Yamamura, *The role of macrophages in antigen presentation and T cell tolerance.* Int Immunol, 1993. 5: p. 1023-1033.

184. Aiba, Y., *et al.*, *Requirement of a second signal from antigen presenting cells in the clonal deletion in immature T cells.* Int Immunol, 1994. 6: p. 1475-1483.

185. Page, D.M., *et al.*, *Two signals are required for negative selection of CD4+8+ thymocytes.* J Immunol, 1993. 151: p. 1868-1880.

186. Jones, L.A., *et al.*, *CD28-B7 interactions are not required for intrathymic clonal deletion.* Int Immunol, 1993. 5: p. 503-512.

187. Tan, R., *et al.*, *B7 costimulates proliferation of CD4-8+ T lymphocytes but is not required for the deletion of immature CD4+8+ thymocytes.* J Immunol, 1992. 149: p. 3217-3224.

188. Killeen, N., S.G. Stuart, and D.R. Littman, *Development and function of T cells in mice with a disrupted CD2 gene.* EMBO J, 1992. 11: p. 4329-4336.

189. Roberts, J.L., S.O. Sharrow, and A. Singer, *Clonal deletion and clonal anergy in the thymus induced by cellular elements with different radiation sensitivities.* J Exp Med, 1990. 171: p. 935-940.

190. Schonrich, G., *et al.*, *Anergy induced by thymic epithelium.* Eur J Immunol, 1992. 22: p. 1687-1691.

191. Speiser, D.E., *et al.*, *Clonal deletion induced by either radioresistant thymic host cells or lymphohaemopoietic donor cells at different stages of class I-restricted T cell ontogeny.* J Exp Med, 1992. 175: p. 1277-1283.

192. Burkly, L.C., *et al.*, *Clonal deletion of Vβ5+ T cells by transgenic I-E restricted to thymic medullary epithelium.* J Immunol, 1993. 151: p. 3954-3960.

193. Arase, H., *et al.*, *Clonal elimination of self reactive Vβ6+ T cells induced by H-2 products expressed on thymic radio-resistant components.* Int Immunol, 1992. 4: p. 75-82.

194. Hoffman, M.W., J. Allison, and J.F.A.P. Miller, *Tolerance induction by thymic medullary epithelium.* Proc Natl Acad Sci USA, 1992. 89: p. 2526-2530.

195. Salaun, J., *et al.*, *Thymic epithelium tolerized for histocompatibility antigens.* Science, 1990. 247: p. 1471-1474.

196. Husbands, S.D., *et al.*, *Expression of the major histocompatibility complex class 1 antigens at low levels in the thymus induces T cell tolerance via a non-deletional mechanism.* Eur J Immunol, 1992. 22: p. 2655-2661.

197. Carlow, D.A., S.-J. Teh, and H.-S. Teh, *Altered thymocyte development resulting from expressing a deleting ligand on selecting thymic epithelium.* J Immunol, 1992. 148: p. 2988-2995.

198. Murphy, K.M., A.B. Heimberger, and D.Y. Loh, *Induction by antigen of intrathymic apoptosis of CD4+8+ TCR^{lo} thymocytes in vivo.* Science, 1990. 250: p. 1720-1723.

199. Kishimoto, H., C.D. Surh, and J. Sprent, *Upregulation of surface markers on dying thymocytes.* J Exp Med, 1995. 181: p. 649-655.

200. Ogasawara, J., T. Suda, and S. Nagata, *Selective apoptosis of CD4+8+ thymocytes by the anti-FAS antibody.* J Exp Med, 1995. 181: p. 485-491.

201. Strasser, A., *et al.*, *Positive and negative selection of T cells in T-cell receptor transgenic mice expressing a bcl-2 transgene.* Proc Natl Acad Sci USA, 1994. 91: p. 1376-1380.

202. Strasser, A., A.W. Harris, and S. Cory, *Bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship.* Cell, 1991. 67: p. 889-899.

203. Siegel, R.M., *et al.*, *Inhibition of thymocyte apoptosis and negative antigenic selection in bcl-2 transgenic mice.* Proc Natl Acad Sci USA, 1992. 89: p. 7003-7007.

204. Sentman, C.L., *et al.*, *Bcl-2 inhibits multiple forms of apoptosis but not -ve selection in thymocytes.* Cell, 1991. 67: p. 879-888.

205. Yeoman, H. and A.L. Mellor, *Tolerance and MHC restriction in transgenic mice expressing a MHC class I gene in erythroid cells.* Int Immunol, 1992. 4: p. 59-65.

206. Morahan, G., J. Allison, and J.F.A.P. Miller, *Tolerance of class I histocompatibility antigens expressed extrathymically.* Nature, 1989. 339: p. 622-624.

207. Morahan, G., *et al.*, *Expression in transgenic mice of class I histocompatibility antigens controlled by the*

metallothionein promoter. Proc Natl Acad Sci USA, 1989. 86: p. 3782-3786.

208. Morahan, G., M.W. Hoffmann, and J.F.A.P. Miller, *A nondeletional mechanism of peripheral tolerance in T-cell receptor transgenic mice.* Proc Natl Acad Sci USA, 1991. 88: p. 11421-11425.

209. Allison, J., *et al.*, *Diabetes in transgenic mice resulting from over-expression of class I histocompatibility molecules in pancreatic β cells.* Nature, 1988. 333: p. 529-533.

210. Miller, J.F.A.P., *A transgenic window on peripheral T cell tolerance.* Immunol Cell Biol, 1992. 70: p. 49-50.

211. Fields, L.E. and D.Y. Loh, *Organ injury associated with extrathymic induction of immune tolerance in doubly transgenic mice.* Proc Natl Acad Sci USA, 1992. 89: p. 5730-5734.

212. Schonrich, G., *et al.*, *Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction.* Cell, 1991. 65: p. 293-304.

213. Schonrich, G., *et al.*, *Distinct mechanisms of extrathymic T cell tolerance due to differential expression of self antigen.* Int Immunol, 1992. 4: p. 581-590.

214. Alferink, J., *et al.*, *Long life span of tolerant T cells and the role of antigen in maintenance of peripheral tolerance.* Int Immunol, 1995. 7: p. 331-336.

215. Ferber, I., *et al.*, *Levels of peripheral T cell tolerance induced by different doses of tolerogen.* Science, 1994. 263: p. 674-676.

216. Schonrich, G., *et al.*, *Tolerance induction as a multi-step process.* Eur J Immunol, 1994. 24: p. 285-293.

217. Arnold, B., G. Schonrich, and G.J. Hammerling, *Multiple levels of peripheral tolerance.* Immunol Today, 1993. 14: p. 12-17.

218. Mann, D.W., *et al.*, *Cytotoxic T lymphocytes from mice with soluble class I Q10 molecules in their serum are not tolerant to membrane-bound Q10.* J Immunol, 1987. 138: p. 240-245.

219. Wieties, K., *et al.*, *Peripheral tolerance in mice expressing a liver-specific class I molecule: inactivation/deletion of a T-cell subpopulation.* Proc Natl Acad Sci USA, 1990. 87: p. 6604-6608.

220. Jones-YoungBlood, S.L., *et al.*, *Effect of the expression of a hepatocyte-specific MHC molecule in transgenic mice on T cell tolerance.* J Immunol, 1990. 144: p. 1187-1195.

221. Rocha, B. and H. von Boehmer, *Peripheral selection of the T cell repertoire.* Nature, 1991. 251: p. 1225-1228.

222. Rocha, B., C. Tanchot, and H. von Boehmer, *Clonal anergy blocks in vivo growth of mature T cells and can be reversed in the absence of antigen.* J Exp Med, 1993. 177: p. 1517-1521.

223. Zhang, L., *et al.*, *Role of infused CD8+ cells in the induction of peripheral tolerance.* J Immunol, 1994. 152: p. 2222-2228.

224. Zhang, L., *et al.*, *Peripheral deletion of mature CD8+ antigen-specific T cells after in vivo exposure to male antigen.* J Immunol, 1992. 148: p. 3740-3745.

225. Sarvetnick, N., *et al.*, *Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma.* Cell, 1988. 52: p. 773-782.

226. Gotz, J., H. Eibel, and G. Kohler, *Non-tolerance and differential susceptiblity to diabetes in transgenic mice expressing major histocompatibility class II genes on pancreatic β cells.* Eur J Immunol, 1990. 20: p. 1677-1683.

227. Burkly, L.C., D. Lo, and R.A. Flavell, *Tolerance in transgenic mice expressing major histocompatibility molecules extrathymically on pancreatic cells.* Science, 1990. 248: p. 1364-1368.

228. Burkly, L.C., *et al., T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E.* Nature, 1989. 342: p. 564-566.

229. Markmann, J., *et al., Antigen presenting function of class II MHC expressing pancreatic beta cells.* Nature, 1988. 336: p. 476-479.

230. Miller, J., *et al., Tissue-specific expression of allogeneic class II MHC molecules induces neither tissue rejection nor clonal inactivation of alloreactive T cells.* J Immunol, 1990. 144: p. 334-341.

231. Murphy, K.M., *et al., Peripheral tolerance to allogeneic class II histocompatibility antigens expressed in transgenic mice: Evidence against a clonal-deletion mechanism.* Proc Natl Acad Sci USA, 1989. 86: p. 10034-10038.

232. Bohme, J., *et al., Transgenic mice with I-A on islet cells are normoglycemic but immunologically intolerant.* Science, 1989. 244: p. 1179-1183.

233. Lo, D., *et al., Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells.* Cell, 1988. 53: p. 159-168.

234. Lo, D., *et al., Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells.* J Exp Med, 1989. 170: p. 87-104.

235. Geinger, T., L.R. Gooding, and R.A. Flavell, *T-cell responsiveness to an oncogenic peripheral protein and spontaneous autoimmunity in transgenic mice.* Proc Natl Acad Sci USA, 1992. 89: p. 2985-2989.

236. Hanahan, D., *Heritable formation of pancreatic β-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogene.* Nature, 1985. 315: p. 115-122.

237. Ye, X., *et al., Timely immunization subverts the development of peripheral nonresponsiveness and suppresses tumor development in simian virus 40 tumor antigen-*

transgenic mice. Proc Natl Acad Sci USA, 1994. 91: p. 3916-3920.

238. Adams, T.E., S. Alphert, and D. Hanahan, *Non-tolerance and autoantibodies to a transgenic self antigen expressed in pancreatic β cells.* Nature, 1987. 325: p. 223-228.

239. Ohashi, P.S., *et al.*, *Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice.* Cell, 1991. 65: p. 305-317.

240. Kyburz, D., *et al.*, *T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides.* Eur J Immunol, 1993. 23: p. 1956-1962.

241. Bachmann, M.F., *et al.*, *T helper cell unresponsiveness: rapid induction in antigen-transgenic and reversion in non-transgenic mice.* Eur J Immunol, 1994. 24: p. 2966-2973.

242. Lo, D., *et al.*, *Peripheral tolerance to an islet cell-specific hemagglutinin transgene affects both CD4+ and CD8+ T cells.* Eur J Immunol, 1992. 22: p. 1013-1022.

243. Roman, L.M., *et al.*, *The expression of the influenza virus hemagglutinin in the pancreatic β cells of transgenic mice results in autoimmune diabetes.* Cell, 1990. 61: p. 383-396.

244. Sarvetnick, N., *et al.*, *Loss of pancreatic islet tolerance induced by β-cell expression of interferon-gamma.* Nature, 1990. 346: p. 844-847.

245. Allison, J., *et al.*, *Inflammation but not autoimmunity occurs in transgenic mice expressing constitutive levels of interleukin-2 in islet β cells.* Eur J Immunol, 1992. 22: p. 1115-1121.

246. Elliot, E.A. and R.A. Flavell, *Transgenic mice expressing constitutive levels of IL-2 in islet β cells develop diabetes.* Int Immunol, 1994. 6: p. 1629-1637.

247. Guerder, S., *et al.*, *Co-stimulation B7-1 confers antigen-presenting-cell function to parenchymal tissue and in conjunction with tumor necrosis factor alpha leads to*

autoimmunity in trasngenic mice. Proc Natl Acad Sci USA, 1994. 91: p. 5138-5142.

248. Jones, L.A., *et al., Peripheral clonal elimination of functional T cells.* Science, 1990. 250: p. 1726-1728.

249. Webb, S., C. Morris, and J. Sprent, *Extrathymic tolerance of mature T cells: Clonal elimination as a consequence of immunity.* Cell, 1990. 63: p. 1249-1256.

250. Fink, P.J., C.A. Fang, and G.L. Turk, *The induction of peripheral tolerance by the chronic activation and deletion of CD4+Vβ5+ cells.* J Immunol, 1994. 152: p. 4270-4281.

251. Miethke, T., *et al., Exogenous superantigens acutely trigger distinct levels of peripheral T cell tolerance/immunosuppression: dose-response relationship.* Eur J Immunol, 1994. 24: p. 1893-1902.

252. Lussow, A.R. and H. Robson MacDonald, *Differential effects of superantigen-induced "anergy" on priming and effector stages of a T cell-dependent antibody response.* Eur J Immunol, 1994. 24: p. 445-449.

253. Perkins, D.L., *et al., Superantigen-induced peripheral tolerance inhibits T cell responses to immunogenic peptides in TCR (β-chain) transgenic mice.* J Immunol, 1993. 150: p. 4284-4291.

254. Perkins, D.L., *et al., Differential expression of activation markers during tolerance induced by superantigens in T-cell receptor (β-chain) transgenic mice.* Cell Immunol, 1994. 156: p. 310-321.

255. Sundstedt, A., *et al., Superantigens anergize cytokine production but not cytotoxicity in vivo.* Immunology, 1994. 82: p. 117-125.

256. Yui, K., *et al., Two separate mechanisms of T cell clonal anergy to Mls-1^{a1}.* J Immunol, 1993. 151: p. 6062-6075.

257. Bhandoola, A., *et al., Delayed allograft rejection by T cell receptor Vβ8.1 transgenic mice peripherally tolerized to Mls-1^a.* Eur J Immunol, 1994. 24: p. 1710-1713.

258. Bhandoola, A., *et al., Reduced CD3-mediated protein tyrosine phosphorylation in anergic CD4+ and CD8+ T cells.* J Immunol, 1993. 151: p. 2355-2367.

259. Mattingly, J.A. and B.H. Waksman, *Immunologic suppression after oral administration of antigen.* J Immunol, 1978. 121: p. 1878-1883.

260. Kagnoff, M.F., *Effects of antigen-feeding on intestinal and systemic immune responses. iv. similarity between the suppressor factor in mice after erythrocyte-lysate injection and erythrocyte feeding.* Gastroenterology, 1980. 79: p. 54-61.

261. Strobel, S., *et al., Immunological response to fed protein antigens in mice II. Oral tolerance for CMI is due to activation of cyclophosphamide-sensitive cells by gut-processed antigen.* Immunology, 1983. 49: p. 451-456.

262. Strobel, S. and A. Ferguson, *Immune responses to a fed protein antigen in mice. 3. Systemic tolerance or priming is related to age at which antigen in first encountered.* Ped Research, 1984. 18: p. 588-593.

263. Strobel, S. and A. Ferguson, *Modulation of intestinal and systemic immune responses to a fed protein antigen, in mice.* Gut, 1986. 27: p. 829-837.

264. Faria, A.M., *et al., Decrease in susceptibility to oral tolerance induction and occurrence of oral immunization to ovalbumin in 20-38-week-old mice. The effect of interval between oral exposures and rate of antigen intake in the oral immunization.* Immunology, 1993. 78: p. 147-151.

265. Miller, A., *et al., Epitopes of myelin basic protein that trigger TGF-β release after oral tolerization are distinct from encephalitogenic epitopes and mediate epitope-driven bystander suppression.* J Immunol, 1993. 151: p. 7307-7315.

266. Mowat, A.M., *The regulation of immune responses to dietary proteins antigens.* Immunol Today, 1987. 8: p. 93-98.

267. Lamont, A.G., *et al., Genetic control of oral tolerance to ovalbumin in mice.* Immunology, 1988. 63: p. 737-739.

268. Moreau, M.C. and G. Corthier, *Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance to ovalbumin in C3H/HeJ mice.* Infect and Immun, 1988. 56: p. 2766-2768.

269. Melamed, D. and A. Friedman, *Modification of the immune response by oral tolerance: antigen requirements and interaction with immunogenic stimuli.* Cell Immunol, 1993. 146: p. 412-420.

270. Melamed, D. and A. Friedman, *Direct evidence for anergy in T lymphocytes tolerized by oral administration of ovalbumin.* Eur J Immunol, 1993. 23: p. 935-942.

271. Hoyne, G.F., *et al., Differences in epitopes recognized by T cells during oral tolerance and priming.* Immunol Cell Biol, 1994. 72: p. 29-33.

272. Khoury, S.J., W.W. Hancock, and H.L. Weiner, *Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associate with down regulation of inflammatory cytokines and differential up regulation of transforming growth factor β, interleukin 4, and prostaglandin E expression in the brain.* Eur J Immunol, 1992. 176: p. 1355-1364.

273. Whitacre, C.C., *et al., Oral tolerance in experimental autoimmune encephalomyelitis.* J Immunol, 1991. 147: p. 2155-2163.

274. Miller, A., O. Lider, and H.L. Weiner, *Antigen-driven bystander suppression after oral administration of antigens.* J Exp Med, 1991. 174: p. 791-798.

275. Miller, A., *et al., Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor β after antigen-specific triggering.* Proc Natl Acad Sci USA, 1992. 89: p. 421-425.

276. Chen, Y., *et al., Regulatory T cell clones induced by oral tolerance: Suppression of autoimmune encephalomyelitis.* Science, 1994. 265: p. 1237-1240.

277. Gregerson, D.S., W.F. Obritsch, and L.A. Donoso, *Oral tolerance in experimental autoimmune uveoretinitis. Distinct mechanisms of resistance are low dose vs high dose feeding protocols.* J Immunol, 1993. 151: p. 5751-5761.

278. Wang, Z.-Y., *et al., Induction of interferon gamma, interleukin-4 and transforming growth factor- β in rats orally tolerized against experimental autoimmune myasthenia gravis.* Cell Immunol, 1994. 157: p. 353-368.

279. Weiner, H.L., *et al., Oral tolerance: Immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens.* Annu Rev Immunol, 1994. 12: p. 809-837.

280. Melamed, D. and A. Friedman, *In vivo tolerization of Th1 lymphocytes following a single feeding with ovalbumin: anergy in the absence of suppression.* Eur J Immunol, 1994. 24: p. 1974-1981.

281. Hoyne, G.F., *et al., T-cell lymphokine response to orally administered proteins during priming and unresponsiveness.* Immunology, 1993. 78: p. 534-540.

282. Vidard, L., L.J. Colarusso, and B. Bencacerraf, *Specific T-cell tolerance may be preceded by a primary response.* Proc Natl Acad Sci USA, 1994. 91: p. 5627-5631.

283. Fishman-Lobell, J., A. Friedman, and H.L. Weiner, *Different kinetic patterns of cytokine gene expression in vivo in orally tolerant mice.* Eur J Immunol, 1994. 24: p. 2720-2724.

284. Friedman, A. and H.L. Weiner, *Induction of anergy or active suppression following oral tolerance is dertermined by antigen dosage.* Proc Natl Acad Sci USA, 1994. 91: p. 6688-6692.

285. Migita, K. and A. Ochi, *Induction of clonal anergy by oral administration of staphylococcal enterotoxin B.* Eur J Immunol, 1994. 24: p. 2081-2086.

286. Strobel, S. and A. Ferguson, *Persistence of oral tolerance in mice fed ovalbumin is different for humoral and cell-mediated immune responses.* Immunology, 1987. 60: p. 317-318.

287. Peng, H.-J., M.W. Turner, and S. Strobel, *The generation of a 'tolerogen' after the ingestion of ovalbumin is time-dependent and unrelated to serum levels of immunoreactive antigen.* Clin Exp Immunol, 1990. 81: p. 510-515.

288. Mowat, A.M. and D.M.V. Parrott, *Immunological responses to fed protein antigens in mice IV. Effects of stimulating the reticuloendothelial system on oral tolerance and intestinal immunity to ovalbumin.* Immunology, 1983. 50: p. 547-554.

289. Mowat, A.M., A.G. Lamont, and D.M.V. Parrott, *Suppressor T cells, antigen-presenting cells and the role of I-J restriction in oral tolerance to ovalbumin.* Immunology, 1988. 64: p. 141-145.

290. Mowat, A.M., *Depletion of suppressor T cells by 2'-deoxyguanosine abrogrates tolerance in mice fed ovalbumin and permits the induction of intestinal delayed-type hypersensitivity.* Immunology, 1986. 58: p. 179-184.

291. Johnson, J.G. and M.K. Jenkins, *The role of anergy in peripheral T cell unresponsiveness.* Life Sciences, 1994. 55: p. 1767-1780.

292. Aroeira, L.S., *et al.*, *Anti-IL-10 treatment does not block either the induction or the maintenance of orally induced tolerance to Ova.* Scand J Immunol, 1995. 41: p. 319-323.

293. Simons, J.P., M. McClenaghan, and A.J. Clark, *Alternation of the quality of milk by expression of sheep β-lactoglobulin in transgenic mice.* Nature, 1987. 326: p. 530-532.

294. Jenness, R., *Biosynthesis and composition of milk.* J Invest Dermatol, 1974. 63: p. 109-118.

295. Clark, A.J., *et al.*, *Pharmaceuticals from transgenic livestock.* TIBTECH, 1987. 5: p. 20-24.

296. Bell, K. and H.A. McKenzie, *β-Lactoglobulins.* Nature, 1964. 204: p. 1275-1279.

297. Godovac-Zimmermann, J., *The structural motif of β-Lactoglobulin and retinol-binding protein: a basic framework for binding and transport of small hydrophobic molecules ?* TIBS, 1988. 13: p. 64-66.

298. Phillips, N.I., R. Jenness, and E.B. Kalan, *Immunochemical comparison of β-Lactoglobulins.* J Immunol, 1968. 100: p. 307-315.

299. McDougall, E.I. and J.C. Stewart, *The whey proteins of the milk of Red Deer (Cerus elaphusL). A homologue of bovine β-Lactoglobulin.* Biochem J, 1976. 153: p. 647-655.

300. Perez, M.D., *et al., Interaction of fatty acids with β-Lactoglobulin and albumin from ruminant milk.* J Biochem, 1989. 106: p. 1094-1097.

301. Jenness, J., *Interspecies comparison of milk proteins.,* in *Developments in Dairy Chemistry-1,* P.F. Fox, Editor. 1982, Applied Sciences Publishers Ltd: London. p. 87-114.

302. Sawyer, L., *et al., Structure and function of bovine β-lactoglobulin.* Biochem Soc Trans, 1985. 13: p. 265-266.

303. Papiz, M.Z., *et al., The structure of β-Lactoglobulin and its similarity to plasma retinol-binding protein.* Nature, 1986. 324: p. 383-385.

304. McKenzie, H.A. and M.H. Sawyer, *Zone electrophoresis of β-Lactoglobulin.* Nature, 1966. 212: p. 161-163.

305. Monaco, H.L., *et al., Crystal structure of the trigonal form of bovine beta-lactoglobulin and of its complex with retinol at 2.5 A resolution.* J Mol Biol, 1987. 197: p. 695-706.

306. Ali, S. and A.J. Clark, *Characterisation of the gene encoding ovine beta-lactoglobulin. Similarities to the genes for retinol binding protein and other secretory proteins.* J Mol Biol, 1988. 199: p. 415-426.

307. Kanai, M., A. Raz, and D.S. Goodman, *Retinol-binding protein: the transport protein for Vitamin A in human plasma.* J Clin Invest, 1968. 47: p. 2025-2043.

308. Futterman, S. and J. Heller, *The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, β-Lactoglobulin, and the retinol-binding protein of human plasma.* J Biol Chem, 1972. 247: p. 5168-5172.

309. Sundelin, J., *et al.*, *The primary structure of bovine cellular retinoic acid-binding protein.* J Biol Chem, 1985. 260: p. 6494-6499.

310. Harris, S., *et al.*, *Developmental regulation of the sheep β-Lactoglobulin gene in the mammary gland of transgenic mice.* Developmental Genetics, 1991. 12: p. 229-307.

311. Harris, S., *et al.*, *Gene expression in the mammary gland.* J Reprod Fert, 1990. 88: p. 707-715.

312. Armstrong, J.M., H.A. McKenzie, and W.H. Sawyer, *On the fractionation of β-Lactoglobulin and α-lactabumin.* Biochima et. Biophysica. Acta., 1967. 147: p. 60-72.

313. Lowry, O.H., *Protein measurment with the folin phenol reagent.* J Biol Chem, 1951. 193: p. 265-275.

314. Peterson, G.L., *A simplification of the protein assay method of Lowry et al which is more generally applicable.* Anal Biochem, 1977. 83: p. 346-356.

315. Bensadoun, A. and D. Weinstein, *Assay of proteins in the presence of interfering materials.* Anal Biochem, 1976. 70: p. 241-250.

316. Chandler, H.M., *et al.*, *An investigation of the use of urease-antibody conjugates in enzyme immunoassay.* J Immunol Methods, 1982. 53: p. 187-194.

317. Kawasaki, E.S., *Sample preparation from blood, cells, and other fluids,* in *PCR Protocols. A guide to methods and applications,* M.A. Innis, *et al.*, Editors. 1990, Academic Press, INC: San Diego, California. p. 146-152.

318. Winberg, G., *A rapid method for preparing DNA from blood, suited for PCR screening of transgenes in mice.* PCR Methods and Applications, 1991. 1: p. 72-74.

319. Levine, B.B., A. Ojeda, and B. Benaceraff, *Studies on artificial antigens. (III) The genetic control of the immune response to hapten-poly-L-lysine conjugates in guinea pigs.* J Exp Med, 1963. 118: p. 953-957.

320. Benacerraf, B., I. Green, and W.E. Paul, *The immune response of guinea pigs to hapten-poly-L-lysine conjugates as an example of the genetic control of the recognition of antigenicity.* Cold Spring Harb Symp Quant Biol, 1967. 32: p. 569-575.

321. Bluestein, H., I. Green, and B. Benacerraf, *Specific immune response genes of the guinea pig. (I) Dominant genetic control of immune responsiveness to co-polymers of L-glutamic acid and L-alanine and L-glutamic acid and L-tyrosine.* J Exp Med, 1971. 134: p. 458-470.

322. McDevitt, H.O. and A. Sela, *Genetic control of the antibody response. (I) Demonstration of determinant-specific differences in response to synthetic polypeptide antigens in two strains of inbred mice.* J Exp Med, 1965. 122: p. 517-531.

323. McDevitt, H.O., *et al.*, *Genetic control of the immune response mapping of the Ir-1 locus.* J Exp Med, 1972. 135: p. 1259-1278.

324. Merryman, C., P.H. Maurer, and D.W. Bailey, *Genetic control of the immune response in mice to a glutamic acid, lysine, phenylalanine co-polymer. (II) Use of recombinant inbred strains of mice to establish association of immune response genes with H-2 genotype.* J Immunol, 1972. 108: p. 937-940.

325. Merryman, C.F. and P.H. Maurer, *Genetic control of immune response against random co-polymers of glutamic acid and alanine (GA) and tyrosine (GT) in inbred mice.* J Immunol, 1976. 116: p. 739-746.

326. Debre, P., *et al., Genetic control of immune suppression. (II) H-2--linked dominant gene control of immune suppression by the random co-polymer L-glutamic acid.* J Exp Med, 1975. 142: p. 1447-1454.

327. Waltenbaugh, C. and S.D. Miller, *Common assays for identifying immune response genes,* in *Genetics and Molecular Immunology,* D.M. Weir, Editor. 1986, Blackwell Scientific Publication: Oxford, Great Britain. p. 103.1-103.9.

328. Carbone, F.R. and M.J. Bevan, *Major Histocompatibility complex control of T cell recognition,* in *Fundamental Immunology,* W.E. Paul, Editor. 1989, Raven Press Ltd: New York. p. 541-567.

329. Titus, R.G. and J.M. Chiller, *A simple and effective method to assess murine delayed type hypersensitivity to protein.* J Immunol Methods, 1981. 45: p. 65-78.

330. Hachimura, S., *et al., Suppression of the systemic immune response to casein by oral administration of a tryptic digest of casein.* Biosci Biotech Biochem, 1993. 57: p. 1674-1677.

331. Enomoto, A., *et al., Milk whey protein fed as a constituent of the diet induced by both oral tolerance and a systemic humoral response, while heat-denatured whey protein induced only oral tolerance.* Clin Immunol Immunopathol, 1993. 66: p. 136-142.

332. Hachimura, S., *et al., Differential inhibition of T and B cell responses to individual antigenic determinants in orally tolerized mice.* Int Immunol, 1994. 6: p. 1791-1797.

333. Kim, S.M., *et al., Serum antibody response elicited by a casein diet is directed to only limited determinants of $\alpha s1$-casein.* Int Arch Allergy Appl Immunol, 1993. 101: p. 260-265.

334. Carr, R.I., *et al., Orally induced tolerance to casein in mice on normal mouse chow.* Clin Immunol Immunopathol, 1986. 40: p. 497-504.

335. Davey, B., *Antigens,* in *Immunology. A foundation text.* 1989, Open University Press: p. 76-88.

336. Takahashi, T., K. Yamauchi, and S. Kaminogawa, *Comparison between the antigenicity of native and unfolded β-Lactoglobulin.* Agric Biol Chem, 1990. 54: p. 691-697.

337. Takahashi, T., *et al., T cell recognition of β-Lactoglobulin.* Agric Biol Chem, 1988. 52: p. 2485-2491.

338. Tsuji, N.M., J.-I. Kurisaki, and K. Mizumachi, *Establishment of CD4+ T cell clones specific to bovine β-Lactoglobulin and analysis of their specificity.* J Biochem, 1993. 113: p. 545-548.

339. Alexander, L.J., *et al., Complete nucleotide sequence of the bovine β-lactoglobulin gene.* Animal Biotechnology, 1993. 4: p. 1-10.

340. Alexander, L.J., *et al., Complete sequence of the bovine β-lactoglobulin cDNA.* Nucleic Acids Res, 1989. 17: p. 6739.

341. Erhardt, G., J. Godovac-Zimmermann, and A. Conti, *Isolation and complete primary sequence of a new ovine wild-type B-Lactoglobulin C.* Biol Chem, 1989. 370: p. 757-762.

342. Cibotti, R., *et al., Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants.* Proc Natl Acad Sci USA, 1992. 89: p. 416-420.

343. Barashi, I., *et al., Ectopic expression of β-lactoglobulin/human serum albumin fusion genes in transgenic mice: hormonal regulation and in situ localization.* Transgenic Research, 1994. 3: p. 141-151.

344. Bayna, E.M. and J.M. Rosen, *Tissue-specific, high level expression of the rat whey acidic protein gene in transgenic mice.* Nucleic Acids Res, 1990. 18: p. 2977-2985.

345. Vilotte, J.-L., *et al., Efficient tissue-specific expression of bovine α-lactalbumin in transgenic mice.* Eur J Biochem, 1989. 186: p. 43-48.

346. Lee, K.-F., *et al.*, *Tissue-specific expression of the rat β-casein gene in transgenic mice.* Nucleic Acids Res, 1988. 16: p. 1029-1041.

347. Maschio, V., *et al.*, *Transgenic mice carrying the guinea-pig α-lactalbumin gene transcribe milk protein genes in their sebaceous glands during lactation.* Biochem J, 1991. 275: p. 459-467.

348. Roberts, B., *et al.*, *Cloning of the goat β-casein-encoding and expression in transgenic mice.* Gene, 1992. 121: p. 255-262.

349. Persuy, M.-A., *et al.*, *High expression of the caprrine β-casein gene in transgenic mice.* Eur J Biochem, 1992. 205: p. 887-893.

350. Wall, R.J., *et al.*, *High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine.* Proc Natl Acad Sci USA, 1991. 88: p. 1696-1700.

351. Archibald, A.L., *et al.*, *High-level expression of biologically active human α1-antitrypsin in the milk of transgenic mice.* Proc Natl Acad Sci USA, 1990. 87: p. 5178-5182.

352. Dobrovolsky, V.N., *et al.*, *Human γ-interferon expression in the mammary gland of transgenic mice.* FEBS, 1993. 319: p. 181-184.

353. Shani, M., *et al.*, *Expression of human serum albumin in the milk of transgenic mice.* Transgenic Research, 1992. 1: p. 195-208.

354. Lee, K.-F., S.H. Atiee, and J.M. Rosen, *Differential regulation of rat β-casein-chloramphenicol acetyltransferase fusion gene expression in transgenic mice.* Mol Cell Biol, 1989. 9: p. 560-565.

355. Andres, A.-C., *et al.*, *Ha-ras oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation, and tumor induction in transgenic mice.* Proc Natl Acad Sci, 1987. 84: p. 1299-1303.

356. Gunzburg, W.H., *et al., A mammary-specific promoter directs expression of growth hormone not only to the mammary gland, but also to bergmann glia cells in transgenic mice.* Mol Endo, 1991. 5: p. 123-133.

357. Pittius, C.W., *et al., A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice.* Proc Natl Acad Sci USA, 1988. 85: p. 5874-5878.

358. Chisari, F.V., *et al., A transgenic mouse model of the chronic hepatitis B surface antigen carrier state.* Science, 1985. 230: p. 1157-1160.

359. Oldstone, M.B.A., *et al., Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response.* Cell, 1991. 65: p. 319-331.

360. Jolicoeur, C., D. Hanahan, and K.M. Smith, *T-cell tolerance toward a transgenic B-cell antigen and transcription of endogenous pancreatic genes in thymus.* Proc Natl Acad Sci USA, 1994. 91: p. 6707-6711.

361. Arnold, B., *et al., Alloreactive immune responses of transgenic mice expressing a foreign transplantation antigen in a soluble form.* Proc Natl Acad Sci USA, 1988. 85: p. 2269-2273.

362. Cher, D.J. and T.R. Mosmann, *Two types of murine helper T cell clone II Delayed-type hypersensitivity is mediated by Th1 clones.* J Immunol, 1987. 138: p. 3688-3694.

363. Grun, J.L. and P.H. Maurer, *Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in prloiferative responses.* Cell Immunol, 1989. 121: p. 134-145.

364. Bromford, R., *The comparative selectivity of adjuvants for humoral and cell-mediated immunity II. Effect on delayed-type hypersensitivity in the mouse and guines pig, and cell-mediated immunity to tumor antigens in the mouse of freund's incomplete and complete adjuvants, alhydrogel,*

Corynebacterium Parvum, Bordetella Pertussis , muramyl dipeptide and saponin. Clin Exp Immunol, 1980. 39: p. 435-441.

365. Peterson, J.D., *et al., Split tolerance of Th1 and Th2 cells in tolerance to Theiler murine encephalomyelitis virus.* Eur J Immunol, 1993. 23: p. 46-55.

366. Karpus, W.J., J.D. Peterson, and S.D. Miller, *Anergy in vivo: down-regulation of antigen-specific CD4+ Th1 but not Th2 cytokine responses.* Int Immunol, 1994. 6: p. 721-730.

367. Wit, D.D., *et al., The injection of deaggregated gamma globulins in adult mice induces antigen-specific unresponsiveness of T helper type 1 but not type 2 lymphocytes.* J Exp Med, 1992. 175: p. 9-14.

368. Gilbert, K.M., K.D. Hoang, and W.O. Wegle, *Th1 and Th2 clones differ in their response to a tolerogenic signal.* J Immunol, 1990. 144: p. 2063-2071.

369. Lee, C.S., E. Meeusen, and M.R. Brandon, *Local immunity in the mammary gland.* Vet Immunol Immunopathol, 1992. 32: p. 1-11.

370. Fitzpartrick, J.L., *et al., MHC class II expression in the bovine mammary gland.* Vet Immunol Immunopathol, 1992. 32: p. 13-23.

371. Hanson, L.A., *The mammary gland as an immunological organ.* Immunol Today, 1982. 3: p. 168-172.

372. Ryelandt, M., *et al., Lack of T cell tolerance in mice exposed to a protein antigen through lactation.* Cell Immunol, 1995. 162: p. 89-96.

373. Peng, H.J., M.W. Turner, and S. Strobel, *Failure to induce oral tolerance to protein antigens in neonatal mice can be corrected by transfer of adult spleen cells.* Ped Research, 1989. 26: p. 486-490.

374. Hanson, D.G., *Ontogeny of orally induced tolerance to soluble proteins in mice 1. Priming and tolerance in newborns.* J Immunol, 1981. 127: p. 1518-1524.

375. Miller, A., *et al.*, *Orally administered myelin basic protein in neonates primed for immune responses and enhances experimental autoimmune encephalomyelitis in adult animals.* Eur J Immunol, 1994. 24: p. 1026-1032.

376. Halsey, J.F. and D.C. Benjamin, *Induction of immunological tolerance in nursing neonates by absorption of tolerogen from colostrum.* J Immunol, 1976. 116: p. 1204-1207.

377. Piazzon, I., *et al.*, *Transmisson of an MLS-1^{a}-like superantigen to BALB/c mice by foster-nursing on F1 Mls-1^{bxa}mothers.* J Immunol, 1994. 153: p. 1553-1562.

378. Bruce, M.G. and A. Ferguson, *Oral tolerance to ovalbumin in mice: studies of chemically modified and "biologically filtered" antigen.* Immunology, 1866. 57: p. 627-630.

379. Mowat, A.M., *et al.*, *Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide.* Immunology,1981. 45: p. 105-113.

APPENDIX 1 Raw data for the Lowry test

a) OD values (600nm) for BSA controls obtained in the Lowry tests performed for the BLG purifications 2-6.

BSA concentration (mg/ml)

Purification (nos)	50	100	200	300	400
2	0.343	0.697	1.200	1.464	1.710
3	0.325	0.585	0.976	1.334	1.606
4	0.353	0.600	0.945	1.296	1.600
5	0.332	0.583	1.014	1.394	1.624
6	0.395	0.610	0.993	1.300	1.498

b) OD values for BSA controls obtained in the Lowry test performed for the first BLG purification.

BSA concentration (mg/ml)

Purification (nos)	80	160	240	320	400
1	0.498	0.829	1.154	1.406	1.718

c) OD values for BLG samples obtained in the Lowry test performed for BLG purification 1-6

BLG concentration (mg/ml)

Purification (nos)	62.5	125	250	500	1000
1	0.354	0.582	0.916	1.508	>2
2	0.226	0.432	0.744	1.256	1.750
3	0.278	0.523	0.874	1.420	1.606
4	0.270	0.521	0.873	1.384	1.918
5	0.274	0.513	0.879	1.328	1.692
6	0.202	0.376	0.741	1.166	1.466

APPENDIX 2 Solutions for SDS gels and Western blotting

(All reagents and chemicals in the following sections were purchased from SIGMA unless stated)

A) 15 % Polyacrlyamide gels

Solution makes enough for 2 gels.

6.99mls 30% Acrylamide
3.5mls Resolving gel buffer*
5.74mls dH_2O
200µls APS
2.5µls Temed

* Resolving gel buffer= 19.2gs of Tris (pH8.8) and 0.4g of SDS made up to 100mls with dH_2O.

B) Stacking gel solution

Solution makes enough for 2 gels.

0.5mls 30% Acrylamide solution
1.25mls Stacking gel buffer *
50µls APS
7µls Temed

* Stacking gel buffer = 6.06gs of Tris (pH6.8) and 0.4gs of SDS made up in 100mls of dH_2O.

C) Sample buffer

100mls of sample buffer was made up in dH_2O.

25mls Stacking gel buffer
20gs Glycerol
10gs SDS
5mls 20mM (pH7.5) EDTA
10mls Mercaptoethanol

D) SDS page gels electrolysis

5 litres of electrolysis buffer was made up in dH_2O

15.15g Tris

72.05g Glycine

5gs SDS

E) Destaining solution

2 litres of destaining buffer was made up in dH_2O.

10% Methanol (BDH)

10% Acetic Acid (BDH)

F) Transfering/ Blotting Buffer

5 litres of blotting buffer was made up with 4 litres of dH_2O.

15.15gs Tris base

72.05gs Glycine

5gs SDS

1 litre Methanol (BDH)

G) Blocking buffer

200mls PBS

100µls Tween 20

10gs BSA

H) PBS-Tween

2 litres of PBS plus 0.05% Tween 20

I) Tris saline solution

100mls of Tris saline solution was made up in dH_2O.

0.12g Trizma

0.9g NaCl (pH7.4)

APPENDIX 3 ELISA solutions

A) Coating Buffer (pH9.6);

1 litre of coating buffer made up in dH_2O

1.5g	Na_2CO_3	(BDH)
2.93g	$NaHCO_3$	(BDH)
0.2g	NaN_3	

B) Washing buffer (pH7.2):

1 litre of washing buffer made up in dH_2O

1.07g	Na_2HPO (anhyd)	(BDH)
0.39g	$NaH_2PO_4.2H_2O$	(BDH)
8.5g	NaCl	(BDH)
0.05%	Tween 20	

c) Diluting Buffer (pH7.2);

1 litre of diluting buffer made up in dH_2O

1.07g	Na_2HPO (anhyd)	(BDH)
0.39g	$NaH_2PO_4.2H_2O$	(BDH)
8.5g	NaCl	(BDH)
0.05%	Tween 20	
0.01%	NaH_3	
0.25%	BSA	

APPENDIX 4 Solutions for PCR.

A) Tail Buffer:

1 litre of Tail buffer made up in dH_2O

0.3M NaAcetate

10mM TrisHCL (pH7.9)

1mM EDTA (pH8.0)

1% SDS

Tail buffer solution was autoclaved before SDS was added. A10% SDS stock solution was filter sterilised using a 0.22μmillipore filter.

B) Stock Proteinase K

A stock solution of proteinase K of 20mg/ml was made up in sterile dH_2O and stored at -20^{o}C until use. 5μls of this stock solution was added to 0.5mls of tail buffer for tail digestion.

C) Red cell lysate

1 litre of Red cell lysate was made up in dH_20 and autoclaved

155mM NH_4Cl

10mM $KHCO_3$

0.1mM EDTA-Na_2 (pH7.4)

15mls of red cell lysate was used per mouse.

D) 10X PCR mix.

1 litre of 10X PCR buffer was made up in dH_2O.

500mM KCl

100mM Tris-Cl (pH8.3)

15mM $MgCl_2$

0.1% Gelating (BDH)

Solution was autoclaved and stored at -20^{o}C until use.

E) Triphosphate mixture (Boehringer)
250µls of 100mM dATP
250µls of 100mM dTTP
250µls of 100mM dCTP
250µls of 100mM dGTP
250µls of distilled H_2O.
This solution was mixed and aliquoted. Stored at -20°C until used.

F) 5X loading buffer;
1 litre of loading buffer was made up in dH_2O.
15% Ficoll 400
0.05% SDS
20mM EDTA (pH8.0)
0.125% Orange G

G) TAE running buffer;
1 litre of TAE running buffer was made up in dH_2O to give a 50X stock solution.
242g Tris Base
37.2g $Na_2EDTA.2H_2O$ (pH8.5)
57.1ml Glacial Acetic Acid (BDH)

APPENDIX 5 RNA analysis.

Distilled water used had been autoclaved and DEPC treated.

A) 1% Fomaldehyde gels

For each gel :

0.5g Agarose
5mls 10X MEN*
36mls distilled H_2O
18mls Formaldehyde (BDH)
Ethidium Bromide

Agarose, 10X MEN and dH_2O were melted via microwaving before being cooled to 65°C at which time formaldehyde was added. A 2-3mm gel was then poured.

B) 10X MEN* (pH7.2)

1 litre of a 10 times buffer solution was made up in dH_2O.

41.8g MOPS
6.8g $NaAC.3H_2O$
3.72g $EDTA.H_2O$

Gels were run in 10X MEN buffer, 100mls of 10X MEN plus 900mls of dH_2O.

C) Sample Buffer

per 100µls

50µls 100% stock solution of Formamide
17.2µls 35-75% stock solution of Formaldehyde
10µls 10X MEN
22.8µls dH_2O.

D) Northern blotting 20X SSC Buffer (pH7)

1 litre of 20X SSC was made up with dH_2O

175.3g NaCl
88.2g Sodium citrate

E) TE buffer

1 litre of TE buffer was made up in dH_2O.

10mM Tris HCl (pH 7.4)

1mM EDTA (pH8)

F) Denhart solution

50X solution of Denharts' solution was made up in 500mls of dH_2O.

5gs	Ficoll
5gs	PVP
5gs	Bovine serum albumin

APPENDIX 6 SDS mouse diets.

Rat and mouse No1 Maintenance diet and rat and mouse No 3 Breeding diet.

INGREDIENTS	% COMPOSITION for MAINTENANCE DIET	% COMPOSITION for BREEDING DIET
CEREAL PRODUCTS (Wheat) (Barley) (Wheatfeed)	88.5%	64.0%
VEGETABLE PROTEINS (Ext. Soya Bean Meal)	6.0%	16.5%
ANIMAL PROTEINS (**Whey protein**) (*Fish Meal *)	2.5%	15.0%
ENERGY SOURCES (Soya Oil)	0.5%	2.0%
SUPPLEMENTATION (Vitamins) (Major Minerals) (Trace Minerals) (Amino Acids)	2.5%	2.5%

*** Fish meal = Breeding diet only**

Whey protein, 50% of the whey protein is β- Lactoglobulin with approximate concentration of 3.0gs whilst 12% is α-Lactalbumin with approximate concentration of 0.7gs. 5% is Bovine serum albumin, 0.23% is casein and minor proteins and 30% is Immunoglobulins

APPENDIX 7 HARLAN TEKLAD 9608 TRM (Auto) mouse diet

In this diet dried whey protein has been substituted with soyabean oil. The ingredients of this diet are as follows;
barley,
wheat,
maize,
wheat feed,
soyameal,
whitefish meal,
lucerne,
soya oil,
dried brewers yeast,
and minerals and vitamins.

Calculated oil concentration is 5%
Calaulated protein concentration is 18.4%.

APPENDIX 8 Individual T cell responses of CBA/Ca female mice immunised and challenged with different quantities of bovine BLG (see Chapter 3).

EXPT NOS	100 + 100	100 + 50	50 + 100	50 + 50	100 + 2% HAG	50 + 2% HAG
	0.02	0.07	0.10	0.12	0.38	0.16
	0.01	0.07	0.10	0.02	0.44	0.35
	0.05	0.01	0.01	0.07	0.38	0.28
	0.03	0.09	0.03	0.09	0.49	0.47
	0.02	0.11	0.07	0.04	0.29	0.32

APPENDIX 9 Individual T cell responses (mm) for mice immunised and challenged with ovine BLG (see Chapter 3)

i) CBA/Ca mice

EXPT NOS	CBA/Ca males EXPT	CBA/Ca males CONT	CBA/Ca female EXPT	CBA/Ca female CONT
1	0.10	0.04	0.10	0.04
	0.11	0.03	0.17	0.01
	0.21	0.02	0.13	0.00
	0.12	0.00	0.11	0.09
	0.21	0.03	0.07	0.01
	0.18	0.00	0.13	0.03
2	0.15	0.02	0.1	0.03
	0.18	0.01	0.11	0.03
	0.30	0.02	0.09	0.08
	0.41	0.03	0.13	0.02
		0.00		0.03
3			0.23	0.06
			0.20	0.01
			0.17	0.00
			0.08	0.08
			0.13	

2) C57BJ/6

EXPT NOS	C57BJ/6 males EXPT	EXPT NOS	C57BJ/6 males CONT	C57BJ/6 females EXPT	C57BJ/6 females CONT
1	0.49	1	0.04	0.2	0.02
	0.47		0.01	0.22	0.01
	0.55		0.05	0.2	0.02
	0.17		0.01	0.2	0.09
	0.39		0.05	0.21	0.03
	0.1	2	0.01	0.18	0.01
2	0.11		0.02	0.22	0.01
	0.12		0.02	0.18	0.04
	0.12		0.03	0.11	0.02

3) C57BJ/6 X CBA/Ca F1

EXPT NOS	F1 male EXPT	F1 male CONT	EXPT NOS	F1 female EXPT	F1 female CONT
1	0.19	0.08	1	0.39	0.07
	0.16	0.05		0.31	0.12

	0.19	0.04		0.31	0.04
	0.13	0.04		0.28	0.12
	0.22	0.06		0.18	
	0.25	0.05	2	0.25	0.01
	0.34	0.02		0.13	0.01
		0.01		0.09	0.04
		0.01		0.20	0.02
		0.01		0.22	
		0.07			

4) BALB/c

EXPT NOS	BALB/c male EXPT	BALB/c male CONT	BALB/c female EXPT	BALB/c female CONT
1	0.22	0.10	0.22	0.07
	0.34	0.06	0.19	0.07
	0.34	0.07	0.18	0.07
	0.24	0.10	0.11	0.04
	0.36	0.02	0.18	0.04
2	0.11	0.08	0.17	0.01
	0.21	0.08	0.21	0.03
		0.05	0.23	0.01
			0.21	0.07
			0.24	0.01

EXPT = mice challenged with 2% Hag BLG
CONT = mice challenged with saline

APPENDIX 10 Antibody responses of mice either suckled on BLG-containing or "normal" mouse milk. Following weaning mice were maintained on a diet lacking whey protein (see Chapter 4).

i) Non-transgenic mice suckled on BLG-containing milk

M(1)	M(2)	M(3)	F(1)	F(2)	F(3)
2	>11	>11	2	>11	>11
3	>11	>11	2	>11	>11
4	>11	>11	2	>11	>11
5	>11	>11	4	>11	>11
5	>11	>11	5	>11	>11
>5	>11	>11	5	>11	>11
>5	>11	>11	5	>11	>11
>5	9	>11	>5	>11	>11
>5	2	11	>5	>11	>11
			>5	9	>11
			>5	8	11
			7	3	9

ii) Transgenic mice suckled on BLG-containing milk

M(1)	M(2)	M(3)	F(1)	F(2)	F(3)
ND	0	2	ND	>11	10
ND	0	2	0	>11	9
ND	0	2	0	10	8
0	0	3	0	4	7
0	1	3	1	4	5
1	1	3	1	4	5
1	2	4	1	3	4
2	2	5	2	3	4
2	2	5	2	3	4
3	2	5	3	3	3
3	2	5	4	3	3
5	3	2	4	2	3
			>5	2	3
			>5	2	3
			>5	1	0

iii) Non-transgenic mice suckled on "normal" mouse milk

M(1)	M(2)	M(3)	F(1)	F(2)	F(3)
7	>11	>11	8	>11	>11
5	>11	>11	8	>11	>11

>5	>11	>11	4	>11	>11
>5	>11	>11	2	6	>11
>5	10	>11			
4	10	>11			
2	10	>11			
0	3	>11			

iv) Transgenic mice suckled on "normal" mouse milk

M(1)	M(2)	M(3)	F(1)	F(2)	F(3)
5	6	8	ND	6	8
4	2	7	0	6	8
0	0	5	0	6	6
			1	4	6
			1	3	5
			1	3	4
			2	3	4
			2	3	4
			5	2	4
			5	2	2
			>5	1	1
			>5	0	1

M= male mice
F= female mice
(nos)= bleed number
ND= no detectable antibody.

APPENDIX 11 Individual T cell responses (mm) for F1 BLG-transgenic and CBA/Ca immunised with bovine or ovine BLG (see Chapter 5).

i) bovine BLG.

EXPT NOS	CBA (M) EXPT	CBA (M) CONT	CBA (F) EXPT	CBA (F) CONT	F1 (M) EXPT	F1 (M) CONT	F1 (F) EXPT	F1 (F) CONT
1	0.48	0.05	0.34	0.03	0.56	0.00	0.29	0.01
	0.55	0.03	0.24	0.02	0.65	0.02	0.19	0.04
	0.37	0.03	0.59	0.03	0.25		0.44	0.04
	0.48	0.02	0.39		0.27		0.36	0.01
	0.37	0.03	0.03		0.33			
2	0.05	0.02	0.27	0.08				
	0.13	0.01	0.18	0.02				
	0.18	0.01	0.28					
	0.11	0.01						

ii) ovine BLG

EXPT NOS	F1 (M) EXPT	F1 (M) CONT	F1 (F) EXPT	F1 (F) CONT
1	0.07	0.01	0.13	0.01
	0.15	0.04	0.10	0.04
	0.21	0.07	0.05	0.04
	0.21		0.05	0.01
	0.13		0.03	
	0.07			
	0.07			
	0.12			
	0.05			
	0.05			

EXPT = mice challenged with 2% BLG
CONT = mice challenged with saline
M = male mice
F = female mice

APPENDIX 12 Individual T cell responses (mm) for mice suckled on BLG-containing or "normal" mouse milk immunised with bovine or ovine BLG (see Chapter 5).

i) bovine BLG-
a) 2% Hag data

EXPT NOS	T(M) S	NT (M) S	T (F) S	T (F) S	T (M) NS	NT (M) NS	T (F) NS	NT (F) NS
1	0.65	0.28	0.33	0.34	0.66	0.40	0.27	0.22
	0.26	0.41	0.33	0.38	0.47	0.35	0.35	0.28
	0.23	0.48	0.46	0.42	0.30	0.30	0.29	0.20
	0.28	0.4	0.29	0.45	0.34	0.22	0.25	0.38
	0.40	0.59	0.36	0.31	0.38	0.50	0.37	0.12
2	0.37	0.84	0.39	0.28	0.56	0.35	0.51	0.22
	0.47	0.59	0.39	0.31	0.40	0.37	0.21	0.26
	0.34	0.46	0.43	0.68	0.45	0.42	0.13	0.23
	0.46	0.76	0.48	0.24	0.31	0.49	0.39	0.28
	0.56	0.40	0.34	0.28		0.69	0.41	0.40

b) bovine BLG- cont

EXPT NOS	T (M) S	NT (M) S	T (F) S	NT (F) S
1	0.03	0.03	0.09	0.07
	0.05	0.10	0.09	0.02
	0.02	0.05	0.13	0.04
	0.03	0.15	0.11	0.07
	0.04		0.04	0.01

ii) ovine BLG-
a) 2% Hag BLG

EXPT NOS	T (M) S	NT (M) S	T (F) S	NT (F) S	T (M) NS	NT (M) NS	T (F) NS	NT (F) NS
1	0.13	0.27	0.14	0.22	0.19	0.22	0.18	0.08
	0.21	0.18	0.11	0.19	0.14	0.17	0.20	0.10
	0.13	0.26	0.10	0.25	0.13	0.21	0.23	0.24
	0.18	0.24	0.05	0.16	0.12	0.33		0.23
		0.19	0.13	0.26		0.27		

2	0.25	0.47	0.18	0.21	0.15	0.12	0.11	0.22
	0.23	0.23	0.04	0.16	0.13	0.19	0.15	0.11
	0.38	0.14	0.24	0.13	0.22	0.29	0.19	0.17
	0.32	0.17	0.06	0.14	0.13	0.16	0.04	0.13
	0.05	0.23		0.22			0.05	0.03

b) ovine BLG- cont

EXPT NOS	T (M) S	NT (M) S	NT (M) NS	T (F) NS	NT (F) NS	EXPT NOS	NT (F) S
1		0.00	0.02		0.05	1	0.03
		0.08	0.05		0.02		0.04
		0.00	0.03			2	0.04
		0.08	0.02				0.03
		0.00					0.04
2		0.01		0.03	0.02		0.06
		0.04		0.09	0.04		0.01
				0.03	0.06		0.00
				0.03	0.03		

T= BLG-transgenic mice
NT= non-transgenic mice
S= mice suckled on BLG-containing milk
NS= mice suckled on "normal" mouse milk
M = male mice
F = female mice

APPENDIX 13 Individual T cell responses (mm) for mice suckled on either BLG-containing or "normal" mouse milk. Following weaning mice were placed on a diet lacking whey protein (see Chapter 5).

i) ovine BLG

EXPT NOS	T (M) S	NT (M) S	T (F) S	NT (F) S	T (M) NS	NT (M) NS	T (F) NS	NT (F) NS
1	0.11	0.20	0.32	0.27	0.17	0.20	0.25	0.16
	0.35	0.23	0.30	0.22	0.07	0.17	0.15	0.17
	0.16	0.16	0.22	0.21	0.15	0.12	0.22	0.43
	0.11	0.26	0.11	0.21	0.17	0.21	0.32	0.18
	0.06		0.13	0.13	0.19	0.25	0.34	
	0.28		0.16		0.02	0.21	0.31	
	0.28				0.06	0.19	0.18	
	0.05				0.19		0.23	
	0.05				0.15			
2	0.21	0.15	0.22	0.18			0.31	0.30
	0.12	0.17	0.16	0.11			0.22	0.27
	0.19	0.33	0.09	0.08			0.32	
	0.19	0.11	0.08	0.10			0.24	
	0.17	0.23	0.16	0.13				
	0.23	0.30	0.18	0.13				
	0.10	0.15		0.10				
				0.23				
				0.03				
				0.06				

T= transgenic mice
NT= non-transgenic mice
S = mice suckled on BLG-containing milk
NS= mice suckled on "normal" mouse milk
M= male mice
F= female mice

APPENDIX 14 Individual T cell responses (mm) for G7 mice suckled on either BLG-containing or "normal" mouse milk (see Chapter 5).

i) ovine BLG-
a) 2% Hag BLG

EXPT NOS	T (M) S	NT (M) S	T (F) S	NT (F) S	T (M) NS	NT (M) NS	T (F) NS	NT (F) NS
1	0.12	0.09	0.23	0.13	0.22	0.30	0.22	0.21
	0.15	0.24	0.12	0.14	0.22	0.19	0.30	0.38
	0.17	0.20	0.09	0.25	0.10	0.23	0.15	0.25
	0.10	0.16	0.14	0.16	0.16	0.17		0.22
	0.25	0.20	0.06	0.10	0.05	0.12		0.28
	0.20	0.24	0.19	0.18		0.16		0.25
	0.13	0.13	0.11	0.15		0.19		0.23
		0.20		0.15		0.20		0.29
		0.21		0.16		0.14		0.27
						0.13		0.15
						0.23		
2	0.20	0.41	0.15	0.06	0.30	0.03	0.08	0.07
	0.32	0.01	0.19	0.20		0.07	0.24	0.07
		0.10	0.15	0.21		0.9	0.19	0.09
		0.22	0.15	0.01		0.19	0.06	0.05
		0.22	0.09	0.17		0.12		0.23
		0.17		0.15				0.15
		0.05						0.12
								0.02

b) ovine BLG-control

EXPT NOS	T (M) S	NT (M) S	T (F) S	NT (F) S	T (M) NS	NT (M) NS	T (F) NS	NT (F) NS
3	0.03	0.08	0.07	0.06	0.16	0.04	0.02	0.02
	0.02	0.02	0.02	0.05	0.10	0.01	0.01	0.05
	0.01	0.00	0.02	0.06	0.14	0.05	0.05	0.08
	0.00	0.05	0.02	0.02	0.05	0.06	0.05	0.11
	0.05	0.01	0.02	0.10	0.08	0.03	0.08	0.03
		0.05	0.02	0.04		0.05		0.09
		0.09	0.04	0.03		0.05		0.01
		0.05	0.04	0.01				0.03
		0.10	0.00	0.01				
		0.05	0.11	0.05				
			0.03	0.06				

			0.01	0.07				
			0.00	0.12				
			0.07	0.02				
			0.10	0.01				
			0.03	0.04				
			0.02	0.06				
			0.03	0.03				

T= BLG-transgenic mice
NT = non-transgenic mice
S = mice suckled on BLG-containing milk
NS = mice suckled on "normal" mouse milk
M= male mice
F = female mice

APPENDIX 15 Individual T cell responses (mm) for mice immunised with ovine BLG and challenged with 2% Hag bovine BLG (see Chapter 5).

i) Mice either suckled on BLG-containing or "normal" mouse milk.

EXPT NOS	T (M) S	NT (M) S	T (F) S	NT (F) S	T (M) NS	NT (M) NS	T(F) NS	NT (F) NS
1	0.35	0.34	0.19	0.22	0.13	0.27	0.20	0.36
	0.16	0.47	0.14	0.25	0.11	0.24	0.12	0.17
	0.18	0.22	0.17	0.30	0.17	0.26	0.22	0.26
	0.20	0.31	0.17	0.20	0.15	0.12	0.21	
		0.30	0.22		0.08	0.29	0.27	
		0.27	0.12		0.22		0.22	
		0.43	0.29		0.17			
		0.16	0.16		0.30			
		0.37			0.17			
					0.18			

APPENDIX 16 Individual T cell responses (mm) for mice fed bovine BLG (see Chapter 7)

i) W+ diet (50/21)

EXPT NOS	50/21 FEMALE	50/21 MALE
1	0.16	0.24
	0.15	0.25
	0.18	0.15
	0.15	0.31
		0.19
2	0.16	0.16
	0.20	0.26
	0.21	0.18
	0.25	0.21
	0.21	

ii) W+ diet (25/21)

EXPT NOS	25/21 FEMALE	25/21 MALE
1	0.26	0.19
	0.22	0.27
	0.26	0.19
	0.10	
	0.21	
2	0.24	0.17
	0.24	0.10
	0.27	0.19
	0.16	0.25

iii) W+ (25/24)

EXPT NOS	25/24 FEMALE	25/24 MALE
1	0.22	0.21
	0.20	0.25
	0.27	0.23
	0.23	0.31
	0.20	0.29
	0.25	0.23
	0.25	
	0.20	

iv) W- (50/21)

EXPT NOS	50/21 FEMALE	50/21 MALE
1	0.17	0.24
	0.12	0.14
	0.22	0.14
	0.19	0.11
	0.15	0.11
		0.23
		0.21
		0.36
2	0.19	0.31
	0.27	0.28
	0.28	0.25
	0.30	0.25
		0.19

v) W- (25/21)

EXPT NOS	25/21 FEMALE	25/21 MALE
1	0.21	0.19
	0.20	0.25
	0.27	0.28
	0.26	0.29
	0.20	0.28
2	0.39	0.21
	0.24	0.33
	0.23	0.19
		0.32
		0.19

vi) W- (50/24)

EXPT NOS	50/24 FEMALE	50/24 MALE
1	0.33	0.26
	0.30	0.12
	0.32	0.36
	0.33	0.37
		0.23
2		0.38

vii) W- (25/24)

EXPT NOS	25/24 FEMALE	25/24 MALE
1	0.27	0.25
	0.29	0.23
	0.31	0.40
	0.21	0.33
	0.29	0.31
	0.28	

W+ = whey containing diet
W- = whey deficient diet
50/21 = 50mgs of bovine BLG for 21 days
25/21 = 25mgs of bovine BLG for 21 days
50/24 = 50mgs of bovine BLG for 24 hour
25/24 = 25mgs of bovine BLG for 24 hours

APPENDIX 16 cont Individual T cell responses (mm) for control mice fed saline in the BLG feeding experiments (see Chapter 7).

i) W+ and W-

CBA (M) W+	CBA (F) W+	CBA (M) W -	CBA (F) W -
0.25	0.32	0.07	0.35
0.28	0.27	0.13	0.36
0.12	0.32	0.44	0.35
0.24	0.20	0.23	0.38
0.15	0.27	0.33	0.26
0.32	0.38	0.29	0.26
0.44	0.23	0.07	0.30
0.26		0.16	
0.12		0.07	